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MARCH 1953

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JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY
Volume 41, Supplement 1, March 1953

SYMPOSIUM
ON
SOME ASPECTS
OF MICROBIAL METABOLISM

PUBLISHED BY
THE WISTAR INSTITUTE OF ANATOMY AND BIOLOGY
PHILADELPHIA
1953

FOREWORD

Concerning Supplements to the

JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

A large part of the cost of most scientific journals is borne by the subscribers. The publication of lengthy manuscripts in the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY would require a further increase in the subscription price or, alternatively, would reduce the number of pages which could be assigned to other authors who desire to publish articles relating to the many fields of physiology encompassed by this Journal. Accordingly, in the interest of our subscribers, it is necessary to limit the number of pages made available to any author and for the presentation of material dealing with any one subject.

Despite these considerations, certain authors rightly desire to publish the results of their research in an extensive form at a cost which cannot fairly be assessed against the subscribers; certain observations and data may require such a form of publication. To satisfy these desires and needs the Editorial Board of the JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY has approved, on a trial basis, the publication of occasional Supplements to the Journal. The articles published in these Supplements must meet the usual standards of scientific merit; the authors must provide the full cost of publication.

It is hoped that such a Supplement will fulfill the requirements for unique publications and will supply scientific reports of unusual interest to our subscribers at no additional cost to them.

DETLEV W. BRONK

SYMPOSIUM
ON SOME ASPECTS
OF MICROBIAL METABOLISM

GIVEN AT

RESEARCH CONFERENCE
FOR BIOLOGY AND MEDICINE
OF THE
ATOMIC ENERGY COMMISSION

sponsored by

THE BIOLOGY DIVISION
OAK RIDGE NATIONAL LABORATORY

Oak Ridge, Tennessee
April 10, 11, 1952

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INTRODUCTION

The fifth annual Biology Research Conference sponsored by the Biology Division of the Oak Ridge National Laboratory in cooperation with the Division of Biology and Medicine of the Atomic Energy Commission, dealt with "Some Aspects of Microbial Metabolism." This subject was chosen on account of its basic importance for an understanding of the synthesizing mechanisms of living cells and for its possible implications in the study of the mechanisms underlying radiation effects. As in previous conferences, free and open discussions were encouraged. These are reproduced in part.

Dr. Stanley F. Carson of the Biology Division, Oak Ridge National Laboratory was largely responsible for the selection of the program. All the speakers of this conference submitted manuscripts and had an opportunity to correct the discussion.

This symposium is the fifth of the series, all of which have appeared as supplements to this Journal.

ALEXANDER HOLLAENDER

INTRODUCTORY REMARKS ON THE COMPARATIVE BIOCHEMISTRY OF MICROORGANISMS

C. B. VAN NIEL

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As one whose scientific development was so greatly influenced by a 7-year apprenticeship in the laboratory of Professor Albert Jan Kluyver, during the years when the idea of the "unity in biochemistry" was being developed, and the concept of "comparative biochemistry" originated, it is perhaps understandable and excusable that I have become, and still am, an addict to this mode of approach. While realizing that there is today a certain reluctance to take these concepts seriously, it seems to me, nevertheless, that this is due to some misunderstanding on the part of the critics. It should be recognized in the first place that the tendency to oversimplification, always somewhat inherent in scientific thinking, becomes even more exaggerated when the possibility emerges of considering an overwhelming mass of apparently unrelated data from a central viewpoint in such a manner that they become closely interconnected. There may not be many among the participants in this conference who can appreciate the enormous clarification brought about by the enunciation of the "unitarian approach"; for the past two decades it has become so much a part of our thinking that the very fact of its once having been started is no longer taken into account. And this sometimes leads to a certain contempt, through lack of perspective, of what has become the obvious, while the inadequacies of the original concept are used to set off in sharp contrast the "great advances" made since. Let us try not to lose sight of the truth contained in the opening statement of Gilbert Newton Lewis' "Anatomy

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of Science," ('26) so aptly quoted by Kluyver in his London lectures ('31): "The strength of science lies in its naïveté." It does not require much perspicacity to realize that. If at the start we could be fully aware of all the complexities of a problem, we would probably wind up in a state of frustration and despair of ever doing a really good experiment or of ever contributing a truly significant idea.

It is true that some of the views expressed by Kluyver during that most fruitful period of 1925–1935 were indeed oversimplified, and we are beginning to learn that there are several aspects of metabolism that range beyond the possibilities considered in the early days. On the other hand, we might do well to recognize that the fundamental aim of Kluyver's approach has, in the subsequent quarter century, been developed to such a magnificent extent that at present the science of comparative biochemistry pervades most of the important biochemical work.

And that today marks the beginning of an important conference on the biochemistry of microorganisms is certainly another feather in Kluyver's cap. Nobody, I believe, realized as keenly as he the enormous advantages that the microorganisms offer over almost any other type of biological material for the study of comparative biochemical problems. The reason is to be found in the fact that, in many cases, their metabolic processes are so much more clear cut than those in the higher plants and animals, as well as in the possibility of influencing these reactions by very simple procedures in a readily interpretable manner. And last, but not least, it is of great importance for the purpose of comparing metabolic processes that we find peculiarly exaggerated types of metabolism among microorganisms. As examples may be mentioned: incomplete oxidations, as encountered in the acetic acid bacteria, often comprising little more than one- or two-step oxidations; incomplete reductions, as in some of the nitrate-reducing bacteria, yielding nitrite, nitrous oxide, or molecular nitrogen as the final reduction product; "extreme" oxidations or reductions, found in the various types of microbes oxidizing hydro-

carbons, or producing methane from carbon dioxide or hydrogen sulfide from hydrogen sulfate in amounts equivalent to the oxidations performed; the bacterial photosyntheses, significant "variants" of green plant photosynthesis; and the carbon dioxide assimilation process, carried out by a variety of chemosynthetic bacteria. A closer study of such metabolic rarities, representing remnants of or adaptations to special ecological niches, is apt to teach us a great deal about biochemistry in general, and for investigations of this sort a knowledge of the microorganisms and their biochemical potentialities is, of course, a major necessity.

In the early days of the development of "comparative biochemistry" a general survey of the diverse metabolic processes was rather obviously considered as constituting the most significant primary attack. It yielded a vast amount of valuable information on the nature of the products formed by a large variety of microorganisms from many different substrates, on their quantitative relations, and on the changes in over-all results that could be induced by variations in experimental conditions. Such information was then used as the basis for attempts at interpreting the manner in which the different products arise. Here the guiding principle was the thesis that every biochemical event can be resolved into a series of elementary step reactions, each representing a simple case of transfer of one or two hydrogen atoms from one molecule to another.

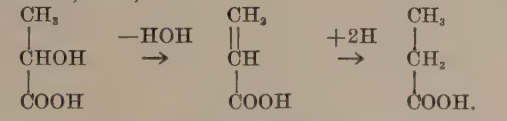
The evidence amassed in this manner indicated that the degradation of a group of closely related substances, such as the sugars, initially proceeded by one single, common pathway; likewise, that the formation of a specific product, wherever encountered, was always accomplished by a common mechanism. In the absence of methods adequate for the detailed investigation of individual step reactions "in isolation," the approach was extremely fruitful. Sometimes, however, the interpretations suffered from the fact that they were taken too seriously, and that an investigation of what appeared to

be unimportant details was neglected. Hence some of the conclusions have appeared to be oversimplifications, based on a desire to show that our understanding of the processes was sufficient to permit the reconstruction of "mechanisms" involving a detailed representation of the component stages. If such an attitude had always been accompanied by a clear recognition that the reconstructions were at best "first approximations," there would not have been much cause for criticism. But occasionally the very fact that an interpretation could be formulated in agreement with the general concepts led to an exaggerated feeling of confidence. "It had to be right," and it did not seem necessary to check certain inferences by experiment.

An instructive example of such a situation is furnished by the studies on the propionic acid fermentation. It had been clearly established by Fitz, von Freudenreich and Orla Jensen, Sherman, and Virtanen (for a detailed discussion and complete bibliography up to 1928, see van Niel, '28) that propionic acid is one of the main end products formed by a fairly well-defined group of bacteria from carbohydrates as well as from lactic and pyruvic acids. The chemical similarity of propionic and lactic acids is obvious; and, because the formation of lactic acid from sugar was of widespread occurrence, and the mechanism of this process reasonably clear, it was concluded that the main problem of the propionic acid fermentation resided in the formulation of a mechanism for the conversion of lactic into propionic acid.

By means of "graphite analysis" this can be easily accomplished; the main principles of comparative biochemistry can be invoked to provide the desired mechanism. Generally, the replacement of a hydroxyl group by hydrogen is accomplished by the elimination of the hydroxyl group as water resulting in a carbon-carbon double bond; the hydrogenation of the dehydration product then yields the desired compound. Applied to the present problem the formation of propionic from lactic

acid can thus be "understood" as proceeding in the following manner (van Niel, '28):



I suspect that it was the very reasonableness of this formulation that made me feel satisfied that the problem had been "solved," and prevented me from doing the one obvious experiment that would then have demonstrated the inadequacy of the solution. This experiment is a test as to the ability of the propionic acid bacteria to carry out a reduction of the unsaturated compound, acrylic acid, to propionic acid. It was not even attempted until 1939, when Kalckar insisted on examining this reaction — with strictly negative results (unpublished; see also Kalckar, '41, p. 95). Even then, the outcome did not disturb me very much; it could always be ascribed to "toxicity" or to some other complication, such as the difference in structure of a substance "in a bottle" and "in an organism."

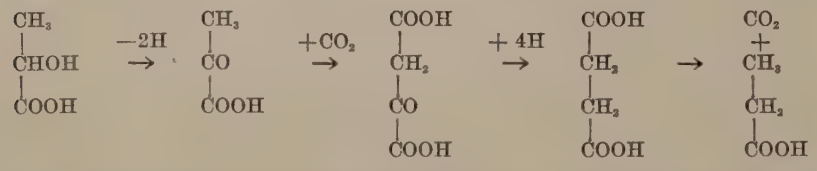
Today we know that the simple mechanism formulated in 1928 cannot account for the facts. The main argument is that Barker and Lipmann ('44) clearly have shown that propionic acid bacteria can produce propionic acid, e.g., from pyruvic acid, under conditions under which lactate is not metabolized at all.

About 20 years after the formulation of a mechanism that does not work, another possibility emerged; it is one that would probably have been dismissed as impossible in 1928, even if it had been considered. It embodies two features quite undreamt of in biochemical philosophy of the 1920's; viz., a reaction in which carbon dioxide is incorporated into organic substances under the influence of heterotrophic bacteria, and another process which represents a decarboxylation of a compound that is not a keto acid. The occurrence of carbon dioxide assimilation by propionic acid bacteria was first established by Wood and Werkman in 1936; improbable as it seemed, the later

experiments with tracer carbon (Carson and Ruben, '40; Wood et al., '41a, b) have amply demonstrated its reality. This discovery has, during the following years, been the impetus for many further studies which have demonstrated that "heterotrophic CO_2-assimilation" is actually of very widespread occurrence (Krebs, '51; Wood, '46; Ochoa, '51); it has been encountered in so many different organisms that nowadays it would be revolutionary even to suggest that there might be one that does not exhibit this phenomenon. At least we have learned!

The second, equally unexpected, development came from the experiments of Alan Johns ('48, '51a) with *Veillonella gazogenes*, an organism which, like the propionibacteria, produces propionic acid from lactic acid and sugars. Cell suspensions of this species were found to convert succinic acid rapidly and completely to carbon dioxide and propionic acid. Later studies with represenatives of the genus *Propionibacterium* by Delwiche ('48) and Johns ('51b) have shown that these bacteria, too, can carry out this kind of decomposition.

Thus there has emerged a possibility for interpreting the formation of propionic acid in a manner that is not subject to the objections mentioned. It can be represented by the following scheme.



We might paraphrase this type of conversion by saying that it represents a mechanism, developed by an organism incapable of reducing lactic or acrylic acid directly, which accomplishes the impossible by using a subterfuge. The deficiency is apparently overcome by adding a handle to the substrate molecule in the form of a second carboxyl group. Hereby is created a molecular species that can readily be reduced by means of a system that is part of the well-nigh universally distributed Krebs cycle complex. The reduction having been smuggled in,

the handle can be eliminated. And the over-all result is a conversion that is indistinguishable from a direct reduction of lactic to propionic acid.

May we now feel confident that the manner of formation of propionic acid is "understood" on a level comparable with that on which we "understand" reactions in organic chemistry? I fear not; there are, namely, cases of propionic acid formation to which the postulated mechanism does not seem to apply because the organisms involved are incapable of decarboxylating succinic acid, and some of them, such as Cardon and Barker's *Clostridium propionicum* ('47), can actually produce propionic acid from acrylic acid.

It is even possible that the decarboxylation of succinic acid is not a simple, one-step process. Our best hope of investigating this reaction in greater detail comes from a recent discovery made by H. R. Whitely (unpublished). She has found that, by growing cultures of *Micrococcus lactilyticus* (another name for *V. gazogenes)* under special conditions, it is possible to obtain cell-free extracts of the bacteria capable of decarboxylating succinic acid. A resolution of this enzyme extract may go far in elucidating the details of the mechanism of the reaction; from preliminary investigations it appears to involve both coenzyme A and cocarboxylase.

At present it would seem reasonable to conclude that there are probably a number of different ways in which propionic acid can be formed, and that we do not yet know the exact details of any one of them. This, I should like to add, represents a step forward from the rather dogmatic stand I took 24 years ago!

Investigations of Whiteley with the group of anaerobic micrococci, of which *V. gazogenes* is a member, have revealed another situation that is of decided significance. These organisms also have the power of decomposing various pyrimidines and purines (Whiteley and Douglas, '51), and of producing propionic acid in some of those processes. Obviously, there are various other kinds of decomposition products formed as well, and the intriguing part of the investigations

relates to the manner in which the nitrogen of the purine, hypoxanthine, is liberated.

This substance, with four nitrogen atoms, distributed evenly over two apparently identical ureide-like linkages, is decomposed by one strain of *M. lactilyticus* with the liberation of 2 moles of ammonia, and 1 mole of urea per mole of substrate. The obvious conclusion must be that the two ureide linkages are physiologically not equivalent. And it would seem important to determine which of the two gives rise to the urea molecule; this should be readily possible by the use of specifically labeled substances, such as 2-C^{14} or 8-C^{14} hypoxanthine. In connection with this problem it may be noted that studies on the microbial degradation of pyrimidines by Lara ('51), Hayaishi and Kornberg ('51), and Wang and Lampen ('52a, b) have indicated the possibility that the ureide group of this type of compound may be eliminated as urea. At an earlier date I would have felt strongly inclined to deduce from these observations that obviously the ureide linkage containing carbon-8 in the purines is the one which is degraded by a preliminary ring opening and subsequent step-by-step decomposition, yielding a molecule of ammonium, then carbon dioxide, and finally a second ammonium molecule. Now I have become wary of such predictions; the only way to find out is to do the crucial experiments.

So far, I have occupied my time by raising questions and problems, and for an introductory paper this may be the most useful approach. I shall continue in this vein, and bring to your attention a situation which, from the point of view of comparative biochemistry, I find as challenging as any.

Among the microorganisms there exist many types that can oxidize any one of a number of simple organic substances, in the presence of oxygen as the final oxidant, and grow at the expense of such oxidations. There are also several kinds of bacteria endowed with the capacity for oxidizing these selfsame compounds in the absence of oxygen, by using nitrate, nitrite, sulfate, or carbon dioxide as the oxidant (or hydrogen acceptor). The organisms that carry out these processes are

known as nitrate- or nitrite-reducing, sulfate-reducing, and carbonate-reducing bacteria. The first group produces variously nitrite, nitrous oxide, or molecular nitrogen as the main reduction product; the second forms hydrogen sulfide; and the third most often methane, although a carbonate reduction to acetic acid is also known to occur.

As far as the oxidation of the organic substrate is concerned, we may suppose that it proceeds primarily under the influence of the same enzyme systems in all cases, that is to say, regardless of the nature of the final hydrogen acceptor. This phase will not be pursued here; the point I wish to make relates to the concomitant reduction of the oxidant. Of course, one can argue that, in a general manner, these reductions, too, are entirely comparable to the reduction of oxygen to water. We merely need to postulate that some organisms can produce a nitrate reductase, nitrite reductase, sulfate reductase, etc., and with the aid of such enzymes couple the substrate oxidation with the reduction of the specific oxidants. If we are ambitious, we could set out on a course that would eventually lead to the isolation and characterization of such enzymes.

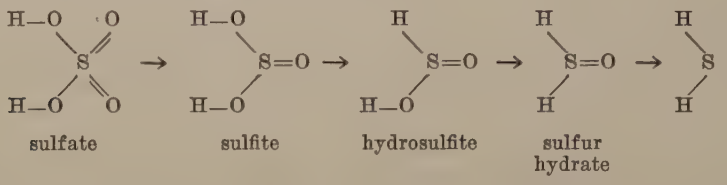
However, this simple approach makes it all the more obvious that there is a problem, and a very fundamental one. This will become clear if we consider the situation in further detail.

There are many organisms, particularly among the microbes, that can live and reproduce themselves in a medium in which sulfate represents the one and only sulfur compound. The mere fact that growth occurs implies that these creatures must be able to manufacture sulfhydryl groups from sulfate, i.e., they must possess a sulfate-reducing enzyme system. The frequency with which artificially produced biochemical mutants that have become dependent on an external supply of reduced sulfur compounds can be isolated effectively underscores the general importance of the sulfate-reducing mechanism and its wide distribution.

If these organisms are, as the evidence shows they must be, in possession of a mechanism for oxidizing substrate molecules, and of a mechanism for reducing sulfate to the sulfhydryl

stage, one should logically expect them to be able to carry out oxidations in the manner of the sulfate-reducing bacteria. Nevertheless, we know that this is not the case, for sulfate reduction, in the sense of a process in which sulfate is used exclusively as the final hydrogen acceptor for the oxidation of substrate, is at present known only as the special property of a very small group of closely related bacteria, the *Desulfovibrio* group, all of them strict anaerobes. Why cannot all bacteria, yeasts, molds, and algae, that can produce SH groups from sulfate carry out the type of metabolism that is so characteristic for *Desulfovibrio*?

The reduction of sulfate to sulfide is almost certainly a process that involves several intermediate reduction stages. The fact that *Desulfovibrio* can use sulfur, hydrosulfite, thiosulfate, and sulfite instead of sulfate as the final oxidant, as was shown by Baars ('30), may be regarded as providing support for this view. Additional and more compelling evidence is furnished by the studies of Fries ('46) with mutants of the mold, *Ophiostoma,* from which it is clear that hexavalent sulfur is converted into divalent sulfur via the intermediate tetravalent stage. Just what the intermediate reduction products are it is impossible to say as yet. At present it does appear, however, that Baars' formulation ('30, p. 97) of sulfate reduction as proceeding by way of



is merely a first approximation, and that the actual intermediate products are likely to be more complex, probably of the nature of organic sulfur compounds. This would be more in line with current concepts such as Phinney's ('48) in which the formation of an early link between sulfate and an amino acid (alanine?), yielding cysteic acid is considered a likely event in the biosynthesis of cysteine, and would imply that

sulfate reduction might more directly involve sulfate esters than inorganic sulfate. In this connection, attention should be called to the fact that a simple means of inducing rapid and copious hydrogen sulfide formation by microorganisms consists in the anaerobic incubation of seaweeds known to contain appreciable amounts of sulfate esters. A detailed microbiological study of these processes is certainly in order; it is well within the realm of possibilities that there exist microorganisms other than *Desulfovibrio* species that can reduce sulfate to hydrogen sulfide provided the former is supplied in an esterified instead of an ionic form.

It is obvious that these cogitations still do not yield an answer to the problem posed at the start. Nevertheless, it is reasonable to expect that comparative studies with different organisms could advance our knowledge of the mechanisms involved in sulfate reduction to the point where a solution to that question will emerge. It should not require emphasizing that in such comparative investigations the most extreme case, as represented by *Desulfovibrio,* must be included.

Before abandoning the topic of sulfur transformations, one other aspect seems worth mentioning. Since the investigations of Trautwein ('24), and particularly of Starkey ('35), it has been known that there are microorganisms capable of oxidizing thiosulfate to tetrathionate. The reverse of this reaction has also been observed. According to the studies of Pollock (Knox and Pollock, '44) it occurs under the influence of an enzyme, tetrathionase, adaptively produced by certain bacterial species. It is now clear that both reactions can be accomplished by the same organism, and that the direction depends upon the conditions. The following experiment, devised by Mr. and Mrs. Baalsrud (personal communication), provides an elegant demonstration.

The organism used is a pure culture of a *Pseudomonas* species, isolated from an enrichment culture in thiosulfate-mineral medium. It is capable of oxidizing thiosulfate to tetrathionate, but it cannot grow at the expense of this oxidation; for growth, the presence of a simple organic compound (e.g.,

glycerol) is needed. A suspension of cells of this organism, incubated in the presence of oxygen, is provided with a measured amount of thiosulfate. A rapid oxygen utilization ensues which abruptly ceases when the thiosulfate has disappeared. The relation between oxygen consumption, thiosulfate disappearance, and alkali production corresponds to that required by the equation,

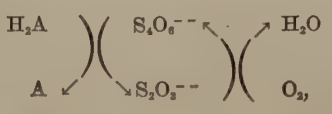
$$2\,Na_2S_2O_3 + H_2O + \frac{1}{2}\,O_2 \rightarrow Na_2S_4O_6 + 2\,NaOH.$$

At this point the atmosphere of the container is replaced by an oxygen-free gas. Under the now anaerobic conditions no measurable changes occur until an organic substrate, which is readily oxidized by the organism in the presence of air, is added. Then tetrathionate is reduced to thiosulfate, concomitantly with the oxidation of the substrate, represented as H_2A:

$$Na_2S_4O_6 + H_2A \rightarrow 2\,NaHS_2O_3 + A.$$

If the amount of substrate added is not in excess of the amount of tetrathionate originally formed, the procedure can be repeated many times. Addition of oxygen will once again cause an oxidation of thiosulfate to tetrathionate, with which another dose of substrate, H_2A, can be oxidized in the absence of oxygen.

In this manner a large amount of organic matter can be oxidized with a small quantity of tetrathionate by the simple expedient of periodically regenerating the latter from its reduction product by temporary exposure to oxygen. The mediating role of the tetrathionate-thiosulfate system, represented in the diagram,



clearly resembles that of the phosphopyridinenucleotides and other coenzymes. In view of the increasing importance assigned to the sulfhydryl groups of enzyme systems, the tetra-

thionate-thiosulfate system may provide us with a simple model for studies on the mechanism of reactions of the type

$$\begin{array}{l} \text{S—H} \\ | \\ \text{R} \end{array} \quad + \quad \begin{array}{l} \text{H—S} \\ | \\ \text{R} \end{array} \quad \begin{array}{l} -2\text{H} \\ \rightleftarrows \\ +2\text{H} \end{array} \quad \begin{array}{l} \text{S—S} \\ | \quad | \\ \text{R} \quad \text{R.} \end{array}$$

As already stated, the case of nitrate reduction presents the same general problem as does sulfate reduction. Many organisms capable of producing amino groups from nitrate (certainly a fargoing reduction!) are unable to bring about oxidations of their normal substrates with nitrate as the final oxidant, under anaerobic conditions. The process of nitrate reduction in the latter sense is much more common among microbes than that of sulfate reduction, and, so far, appears to be associated only with organisms also capable of living aerobically. Another difference in these two reduction processes is that, whereas sulfate reduction is known to yield only hydrogen sulfide, nitrate reduction has been observed with the accumulation of various reduction stages. First among these is nitrite, and some bacteria are limited to this apparently single-step reduction. Others can reduce nitrite, but not nitrate, while a third group can use both nitrate and nitrite as oxidants. In these cases the most commonly encountered reduction products are nitrous oxide and molecular nitrogen. Although, theoretically, a complete reduction to ammonia is conceivable, I do not know of a single instance where the anaerobic oxidation of a substrate is coupled with the quantitative reduction of nitrate or nitrite to ammonia.

Our understanding of the mechanism of the denitrification process has been advanced a little by recent studies carried out in our laboratory (Allen and van Niel, '52). The initial aim of the investigation was to collect evidence for or against the idea that the nitrogen-to-nitrogen link, a necessary step in the formation of nitrous oxide and of nitrogen from nitrite, might be brought about by a reaction of the sort that has long been known to give rise to nitrogen evolution in nonbiological systems, viz.,

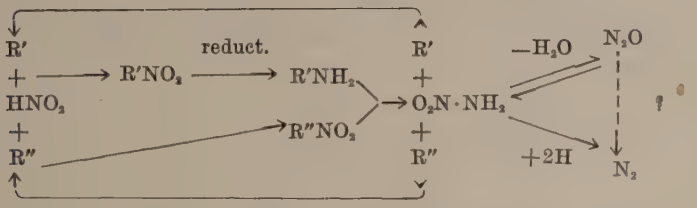
$$\text{RNH}_2 + \text{ONOH} \rightarrow \text{ROH} + \text{N}_2 + \text{H}_2\text{O}.$$

Although the possibility seemed far from remote, the experimental results were completely negative; in no case was the amount of nitrogen produced by the bacteria in excess of that added in the form of nitrite, and this is in spite of conditions that guaranteed a plentiful supply of amino compounds, both in the medium and in the bacteria.

The commonly accepted mechanism for denitrification assumes that nitrite is reduced to hyponitrite, in the dimerized form represented by the formula $H_2N_2O_2$. It is this dimerization that would constitute the manner in which the formation of the nitrogen-nitrogen linkage originates. The production of nitrous oxide would then result from a dehydration, and that of nitrogen from a reduction of the hyponitrous acid. The experimental evidence in support of this postulated mechanism being by no means convincing, tests were carried out with hyponitrite directly. These showed conclusively that, although the substance exerts no inhibitory effects on the formation of nitrogen from nitrite, it is not acted upon by the bacteria. Also nitrous oxide had to be ruled out as the normal precursor of nitrogen because it was found that nitrogen can be produced from nitrite in the presence of cyanide concentrations that completely inhibit the reduction of nitrous oxide.

Further attempts to determine the nature of the precursor to molecular nitrogen led to the discovery that nitramide, $O_2N \cdot NH_2$ or $H-O-\overset{O}{\overset{\diagup\diagdown}{N}}-N-H$, does yield nitrogen under all conditions under which nitrogen is produced from nitrite by the bacteria. From this result it appears reasonable to conclude that the mechanism of denitrification involves an intermediate product which contains two linked nitrogen atoms, one of which is oxidized, and the other reduced. The simplest way to account for the formation of such a substance is by assuming a reaction between a nitro group and an amino group. Postulating, in accordance with the results reported by de la Haba ('50), that nitrite enters into biochemical reactions through the formation of an organic nitro compound, the present status of

our knowledge of the mechanism of the denitrification process can be represented by the following general scheme.



A more detailed analysis of the mechanism naturally requires a precise knowledge of the nature of R′ and R″. It is to be expected that the best approach to studies of this kind will involve an investigation of the enzyme systems operative in the denitrifying bacteria. So far our attempts to prepare from the organisms extracts which can catalyze the reduction of nitrate or nitrite have been unrewarding.

There are several cases on record to support the thesis that a greater comprehension of a particular breakdown process is of significance in permitting the formulation of hypotheses concerning the reverse reaction. This aspect of comparative biochemistry could be applied to the studies on denitrification. In that event it may be anticipated that they might be brought to bear on our understanding of biological nitrogen fixation. To be sure, the latter is not the exact opposite of denitrification; if this were so, nitrogen fixation should lead to nitrite or nitrate formation. This possibility has been adequately disproved by the beautiful studies of Wilson and collaborators (Wilson and Burris, '47). The most convincing argument derives from their observation that assimilation of nitrogen is instantly inhibited by the addition of ammonium to the culture, whereas a similar inhibition by nitrate occurs only after a period of adaptation if the mother culture has been grown in nitrate-free media. This was, I believe, the first case in which enzymatic adaptation was employed as a tool for the study of biochemical mechanisms; soon afterward the technique of simultaneous enzymatic adaptation was developed as a general principle by Stanier ('47).

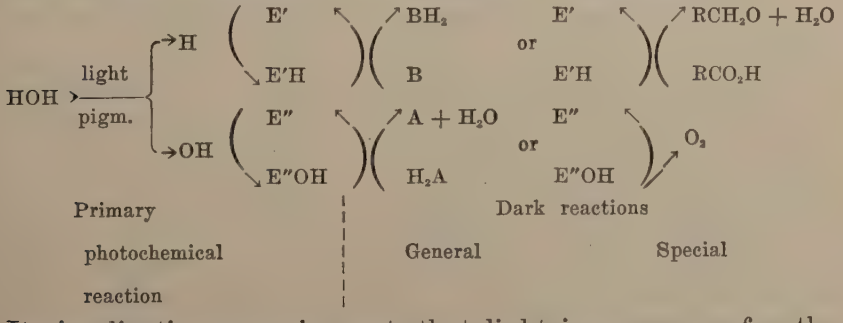
But the fact that nitrate is not directly involved in nitrogen fixation does not necessarily mean that a reversal of the last stages of denitrification cannot represent the mechanism whereby the initial stages of the fixation reaction proceed. By postulating this possibility, it is at once evident that one of the first fixation products would be represented by $R'NH_2$. This type of compound could readily yield, by transamination reactions, substances such as glutamic and aspartic acids, generally recognized as the first detectable products of nitrogen fixation. This would circumvent the need for the intermediate formation of ammonia, and those who are familiar with the literature on biological nitrogen fixation know how completely unsuccessful have been the attempts to procure evidence for the occurrence of ammonia as an intermediate product in this process.

Besides, this postulate carries another implication. The formation of $R'NH_2$ should be accompanied by the simultaneous appearance of $R''NO_2$, containing the second of the two atoms of the nitrogen molecule. This, however, requires an oxidation reaction, which might require the presence of a specific oxidized coenzyme, continuously re-formed from the reduced state in which it should be converted during the transformation of the nitro group into a second amino group. Is it too wild a speculation to consider the possibility that the inhibition of nitrogen fixation by molecular hydrogen might be ascribed to the maintenance of this coenzyme in a reduced state, which would prevent its participation in the primary fixation reaction? I must admit that the inhibition of nitrogen fixation by hydrogen has long puzzled me. It seems too difficult to take seriously the assumption that hydrogen, with its very different molecular structure, could successfully replace nitrogen at an enzyme surface, and thus by simple competition produce the inhibitory effect. Rather would I consider the possibility that the inhibition of nitrogen fixation by hydrogen finds its counterpart, more or less, in the well-known inhibition of denitrification by oxygen. Whatever the merits of these suggestions, they might in any event serve the purpose of stimu-

lating investigations on nitrogen fixation along somewhat different lines from those used in the past. And, after all, it is only by means of experiments that natural science advances.

Meanwhile, the problem as to why so many organisms exist that can reduce nitrate to amino groups, yet are incapable of carrying out a normal substrate oxidation with nitrate as the final acceptor has not been much advanced by these cogitations. It still strikes me as a fundamental and rather formidable question that is worth contemplating.

Another problem that deserves attention is concerned with carbon dioxide assimilation. In photosynthetic organisms this is accomplished with the aid of absorbed radiant energy. The comparative biochemical studies of photosynthesis by green plants and green and purple bacteria have, in the course of time, led to the concept that the primary photochemical reaction results in the decomposition of water, and in the formation of a reduced and an oxidized enzyme system. The reduced enzyme is considered to be the means by which carbon dioxide, after its incorporation into an organic compound, is reduced. The fate of the oxidized enzyme seems to depend on the type of organism doing photosynthesis. In green plants it is presumably the source of oxygen; in the photosynthetic bacteria it must, in order to participate as OH acceptor in the primary photochemical reaction, be continuously reduced by some hydrogen donor, H_2A (van Niel, '49). The following general scheme represents the essence of this view.



Its implications are, in part, that light is necessary for the assimilation of carbon dioxide only insofar as it serves to pro-

duce E′H, the reduced enzyme system. The actual processes of incorporation of carbon dioxide into an organic molecule, RCO_2H, and of reduction of this compound would both be carried out under the influence of enzyme systems whose functioning does not directly depend on the supply of radiant energy; they are dark reactions.

The recent beautiful and imaginative studies of Vishniac and Ochoa ('52) tend strongly to support this contention. They have shown that it is possible to couple the photochemical activities of isolated chloroplasts, by themselves unable to carry out a process of carbon dioxide assimilation, with the activities of special enzymes and their cofactors in such a manner as to accomplish, with the complete system, an actual assimilation and reduction of carbon dioxide. The significance of this work lies in the fact that the enzymes and cofactors were obtained from various microorganisms and animal tissues, and represent entities that carry out their normal catalytic functions without a supply of radiant energy.

This enzymatic reconstruction of a photosynthetic apparatus seems to me of great significance, not merely as a corroboration of the concept that the carbon dioxide assimilation proper can be accomplished by means of enzyme-induced dark reactions once a reducing system has been made available, but also because it is one way in which the detailed mechanism of carbon dioxide incorporation and reduction can be investigated. In order to permit the conclusion that the enzymes and cofactors, which are found by this technique to cause carbon dioxide assimilation, are actually involved in the normal photosynthetic process it will, of course, be necessary to show that in photosynthesizing cells they are present in appreciable amounts, and can function at a rate compatible with that at which photosynthesis proceeds in the intact organism.

Whatever the outcome of such quantitative studies, it will always remain important to amplify our knowledge by other and independent methods. As Kluyver ('31) has emphasized, we cannot hope to obtain more than circumstantial evidence in support of our interpretations of biochemical mechanisms, and

the best way to guard against errors in judgment is to increase the probability of our deductions by an ever-increasing body of information.

At present there are at least two other approaches to a study of the intricate details of the carbon dioxide assimilation process. One of these is based on the expectation that a photosynthesizing cell should be able to use any one of the intermediate products that arise during the successive transformations of carbon dioxide in place of the latter compound, and with a comparable efficiency.

If the chain of reactions involved in the assimilatory mechanism could be represented by an orderly series,

$$CO_2 \rightarrow P_1 \rightarrow P_2 \rightarrow P_3 \rightarrow ----- P_n,$$

it should follow that P_1, P_2, P_3, etc., could serve equally well as carbon dioxide itself for the continued production of cell material. Hence, growth of the test organism in an environment free of carbon dioxide but supplied with one of the intermediate products should proceed at least as rapidly as it does under "normal" conditions of photosynthesis, i.e., at the expense of carbon dioxide.

It is, of course, more reasonable to suppose that here, as in so many other types of metabolism, cyclic mechanisms are operative. Schematically this could be represented by

$$CO_2 + R_1 \rightarrow R_2 \rightarrow R_3 \rightarrow ----- R_n \rightarrow R_1 + \text{product.}$$

Even in that event, substitution of R_1, R_2, R_3, etc., for carbon dioxide should permit the formation of an amount of product corresponding to the amount of intermediate compound added.

Addition of a genuine intermediate product should, moreover, have predictable effects not only on the growth of photosynthetic organisms in the absence of carbon dioxide; it should also influence the rate or amount of oxygen produced during photosynthesis. The conversion of carbon dioxide on the one hand, or of an already partially reduced substance on the other, into products with the same over-all composition must of necessity entail the liberation of different quantities of oxygen.

This, too, is open to experimental verification. Studies of this kind would, therefore, be aimed at the discovery of chemicals that can replace carbon dioxide for growth and for the induction of oxygen production.

Investigations with this "replacement technique" have been carried out independently at the University of Texas by Cramer ('52) and at the Hopkins Marine Station in collaboration with M. B. Allen. So far, they have not yielded results that can be considered promising. The reason may be simply that the compounds used, largely organic acids of various sorts, bear little or no relation to the true intermediate products arising during the course of carbon dioxide assimilation.

Instead of trying to establish the sequence of reactions leading to the transformation of carbon dioxide into photosynthetic products by this indirect method, it is also possible to use a much more direct approach. The latter would aim at the isolation and characterization of the intermediate products themselves. For this type of study the use of $C^{14}O_2$ offers enormous advantages; it has been employed chiefly by Calvin and collaborators at the University of California ('51), and by Gaffron et al. at the University of Chicago ('51). The results so far obtained have shown first of all that the labeled carbon atoms are encountered in a large number of widely different products, even after relatively short periods of photosynthetic activity. This result is in keeping with the early observations made in studies on the distribution of isotopic nitrogen in components of nitrogen-fixing bacteria. In order to obtain more easily interpretable data, the periods of exposure to light and $C^{14}O_2$ have to be very brief indeed (seconds rather than minutes); under such conditions a progressively smaller number of labeled compounds is found. These are reasonably considered as the first products of carbon dioxide assimilation.

A comparison of these substances with those most frequently found as the result of carbon dioxide fixation by heterotrophic organisms shows little if any similarity. This has led to the tendency to regard the assimilation of carbon dioxide in photosynthesis as proceeding by a unique mechanism. It appears to

me that such a conclusion is not yet justified. The main reason for my contention is that the fixation process in heterotrophic organisms is always studied under conditions where a large amount of organic substrate is being metabolized and only a small amount of carbon dioxide fixed. This factor could easily distort the picture by directing the formation of products resulting from carbon dioxide fixation into specific channels.

A much more rigorous comparison between the pathways of carbon dioxide assimilation by photosynthetic and nonphotosynthetic organisms would seem to be provided by studies on $C^{14}O_2$ assimilation under the influence of chemosynthetic organisms, for which carbon dioxide is the only (ultimate) carbon source. Such investigations have not yet been carried out. This most probably may be ascribed to the fact that biochemists are likely to believe that the chemosynthetic bacteria are very hard to handle. I should like to conclude by pointing out that for the trained microbiologist they hold no greater difficulties than molds, yeasts, algae, or the most common heterotrophs. And for truly comparative biochemical studies they have no equals.

DISCUSSION

Chairman CARSON: In connection with the succinic decarboxylase system, has the absence of fumarase and malic dehydrogenase been shown in this system?

VAN NIEL: Oh, no. This is very preliminary work; the real investigation is just beginning.

CARSON: There is always the possibility that the sequence of events could go back around eventually to pyruvic acid, followed by the carboxylation and two stages of reduction, at least until one eliminates the action of fumarase. I wonder if Dr. Krampitz has any evidence on this particular reaction?

KRAMPITZ: From our meager data, we feel that decarboxylation of succinate might proceed by the following mechanism:

1. The anaerobic oxidation of succinate to fumarate with the concomitant reduction of some 3-carbon compound, on the level of oxidation of pyruvate, present in the cells in catalytic amounts to the lactic level.

2. Conversion of fumarate to malate and oxidation of the latter to oxalacetate with concomitant reduction of the catalytic amount of the reduced 3-carbon compound (lactate ?) to propionic acid.

VAN NIEL: By what stages do you conceive of a reduction of lactic acid?

KRAMPITZ: I said some compound first on the *level* of pyruvic acid and then on the *level* of lactate. I cannot define the compounds but the latter may be acrylic acid as you have suggested. Whatever they are does not matter in terms of the present discussion. To continue with the hypothesis, the decarboxylation of oxalacetate yields carbon dioxide and the 3-carbon compound on the level of oxidation of pyruvate. By this mechanism propionic acid and carbon dioxide would be formed from succinate and there would be regeneration of the catalytic amount of oxidized 3-carbon compound.

We felt that, if it were possible to reduce the catalytic quantity of this material which was necessary to initiate the sequence of events referred to, the cells would not have the property of decarboxylating succinate. This hypothesis was tested with *M. lactilyticus*, the organism which will decarboxylate succinate to propionate and carbon dioxide and which contains hydrogenase. If the organisms are first incubated in an atmosphere of hydrogen and then tested for their ability to decarboxylate succinate (under hydrogen), there is approximately 60–70% inhibition of decarboxylation as compared to a similar experiment performed under an atmosphere of helium or nitrogen. Catalytic amounts of methyl viologen (O/R potential, 0.412 volts) increase the degree of inhibition to 100%. These experiments indicate to us that the decarboxylation of succinate is not a simple process but perhaps involves the mechanism just outlined. Hydrogen reduces the 3-carbon precursor of propionate through the action of hydrogenase, and consequently it is impossible to obtain succinate oxidation which is the first step in the process of decarboxylation. Methyl viologen serves as an efficient electron carrier between hydrogenase and the substance to be reduced, hence the increased inhibition of decarboxylation of succinate by hydrogen.

CARSON: Doctor van Niel, do you really think that the use of the chemoautotrophs will very largely simplify this problem inasmuch as they do not oxidize organic material? Is this not perhaps a great oversimplification, since within these organisms there is produced organic material such as structural units, enzymes, or storage materials? Hence, I should think that at all times within these chemoautotrophs, there exist many organic compounds which cannot be far different from those which are found in the heterotrophic organisms.

VAN NIEL: The use of chemoautotrophs will simplify the problem in the same manner as in the case of the green plants. There too we are dealing with organisms that are full of organic matter; and there we can learn from the use of carbon dioxide for very brief periods of time into what molecular species the carbon dioxide is gradually transformed. This is different from the situation in an organism that utilizes an external carbon compound in the normal or abnormal process of making acids, alcohols, and other breakdown products. In this case there may well be a situation in which there is obtained a large amount of intermediate product that can react with carbon dioxide to produce substances not necessarily and primarily involved in the photosynthetic carbon dioxide reduction process.

That is all. It is not that I do not realize that there are organic materials in the chemoautotrophs and it is not that I do not realize that this very situation presents another tremendous problem, and a curious one. Theoretically, it should be possible to feed a chemoautotroph of the obligatory type, not on carbon dioxide, but on an organic type of molecule from which it can carry out the reactions leading to the formation of cell material. Although I wrote about it almost 10 years ago as constituting a main problem, I still do not know what to do about it. There was a time when I thought people had not used the right kind of organic molecules. Further investigations on our part have not been successful.

H. G. WOOD: In discussing comparative biochemistry I wish to express a point of view with which I think Doctor van Niel

will agree that we should study all types of carbon dioxide utilization, including animals and heterotrophic bacteria, and especially those heterotrophic bacteria that make such compounds as acetic acid in which both carbons are from carbon dioxide. It has been shown that in these fermentations there is a carbon-carbon linkage in which both adjacent carbons are from carbon dioxide. It seems to me there is a good chance that something may be found in study of these reactions which may be useful in understanding autotrophism. The important point is that, unlike most heterotrophic utilization of carbon dioxide, there is a synthesis in which adjacent carbons are both from carbon dioxide.

I should also like to bring up one other point. Doctor van Niel indicated that nitrate may not be acting as an electron acceptor in the same way that oxygen is acting because nitrate will not always replace the requirement for oxygen as an electron acceptor. This, it seems, is assuming that there is only one way in which nitrate is utilized. If one assumes there are two ways in which nitrate is utilized, one for example as a nitrogen source through reduction to amino groups, and the other as an electron acceptor similar to oxygen; then even though nitrate is utilized by the organism, it still may not be able to replace oxygen as an electron acceptor. Its utilization in this case may be only via pathways involving the conversion of nitrate to an amino group but not as an electron acceptor in the way that oxygen functions.

VAN NIEL: I am sorry; that was not implied in the statements. The implication was simply that there is a problem, one showing that organisms can reduce nitrate and make amino groups from it; and yet cannot, as the typical nitrate-reducing bacteria can, utilize nitrate as the one and only oxidant for the oxidation of various oxidizable substrates.

LAMPEN: In studies with the pyrimidine-oxidizing organism, Doctor Wang and I did not find any urea accumulating. This organism is urease negative and the only nitrogenous product we can identify is ammonia. We think that oxidation of the pyrimidine ring does not involve a preliminary cleavage into

a 3-carbon and a ureide unit. Doctor Hayaishi is reporting at the Federation meeting on the cleavage of barbituric acid by his organism to yield urea. We have been unable to obtain any anaerobic degradation of barbituric acid. Thus it appears probable that two methods of cleaving the pyrimidine ring exist.

COHEN: Is it necessary to postulate in carbon dioxide reduction that the immediate intermediate is organic? There are of course such things as ketene, carbon monoxide, and carbon suboxide. Has anything been done with those?

VAN NIEL: No, not a thing.

COHEN: There is an assumption that the immediate product is organic?

VAN NIEL: Quite so.

STREHLER: I may have misunderstood Doctor van Niel, but I thought he said that evidence from the California Photosynthesis Laboratories indicates a dichotomy between the pathway of carbon dioxide fixation in heterotrophs and in green plants. I wonder how much of this might be a reflection of an incomplete knowledge of pathways of carbon dioxide reduction in heterotrophs. The reactions on which attention has been centered have been largely the classical pathways of carbon dioxide fixation, while such studies as Horecker and the Calvin group have made seem to correlate very nicely with each other. Might not the formation of ribulose and sedoheptulose as early products of photosynthesis indicate a greater relationship between heterotrophic and autotrophic pathways of carbon dioxide fixation than might have been suspected?

VAN NIEL: The only reason for the statement is that one can find every month or so a new system for the pathway of carbon dioxide in photosynthesis, and that the most recent concepts do not correspond too well with ideas previously derived from studies with nonphotosynthetic organisms. The tendency has been to look upon carbon dioxide fixation in photosynthetic organisms as the result of the addition of a carboxyl group to an organic molecule with the formation of a new acid. I find this hard to correlate with the formulation of Calvin of the

$C_3 + C_4$ linkage. I quite agree with you that we do not know enough about the synthesis of carbon dioxide into organic matter either by nonphotosynthetic or by photosynthetic organisms. In the long run it may well be possible to get to the point where we say, "Oh, well, comparative biochemistry, there it is; it is much the same way in all organisms." But we are not there yet.

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RESPIRATORY CYCLES

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EIGHT FIGURES

The progress made in untangling the complexities of intermediary carbohydrate metabolism during the past decade has been an achievement of which the contemporary biochemist may well be proud. The area of knowledge encompassed by the term "respiratory cycles" has been one of the most rapidly progressing fields. Knowledge available here is so well established and data from different laboratories so nicely in agreement that the entire area may well be considered consolidated. The establishment of this area of agreement, after somewhat extensive controversy, now permits us to look beyond our present knowledge to determine whether there may not exist reactions and processes not yet within experimental proof. It is the purpose of this paper to point out that there are experimental indications of further reactions, particularly in the area of respiratory cycles not yet encompassed by our present knowledge, and it is toward these that further study should be directed.

We should first consider the meaning of the term, "respiratory cycles." It is perhaps characteristic of progress in enzyme chemistry that this term is in general use. From the viewpoint of detailed mechanism it is of utmost importance to separate and purify enzyme systems and to work with these in a stepwise manner. Nevertheless it is also well recognized that in the living cell the reactions are carried out by what have been termed "teams of enzymes" or "enzyme aggregates" and it is apparently characteristic of living cells that

operations are conducted by a cyclic process. We may separate the steps of such a cyclic process but, in so doing, we usually find several places in which the product of a given reaction inhibits either that reaction or one somewhere else in the cycle. In fact, upon consideration of what must happen in the living cell where presumably all the enzymes in a cycle are operating simultaneously, it is apparent that the progress of a quantity of a given compound is not quantitatively stepwise but almost a molecule at a time. This is a characteristic of such cycles in living cells and we shall point out some of its significant features later.

For purposes of this discussion, we shall take the term "respiratory cycle" to mean those enzyme systems wherein the end products of carbohydrate fermentation are converted principally into carbon dioxide and water. Furthermore, we shall assume as proven that the end product of carbohydrate fermentation is pyruvate. This pyruvate enters into a variety of reactions, most of which are illustrated in figure 1. One set of these reactions, the citric acid cycle, which brings about the complete oxidation of pyruvate to carbon dioxide and water, is an example of a respiratory cycle. Knowledge of such a system consists of information on two types of processes, one of which deals with the reactions of the carbon of the pyruvate as illustrated in the figure and the other involves knowledge of the pathway of hydrogen to molecular oxygen, the respiratory system proper. In the present discussion, we shall ignore the details of the pathway of hydrogen or electrons to oxygen, making the generalization that such pathways exist and that they normally involve the participation of several heavy metal pigments, the cytochromes. While this area is a most important part of the respiratory cycles, we shall concentrate upon the carbon transformations, recognizing that the electrons released are carried by appropriate systems to oxygen or other "hydrogen acceptors," depending upon the circumstances.

Considering, then, the transformations of carbon portion of pyruvate in the respiratory cycles resulting in carbon diox-

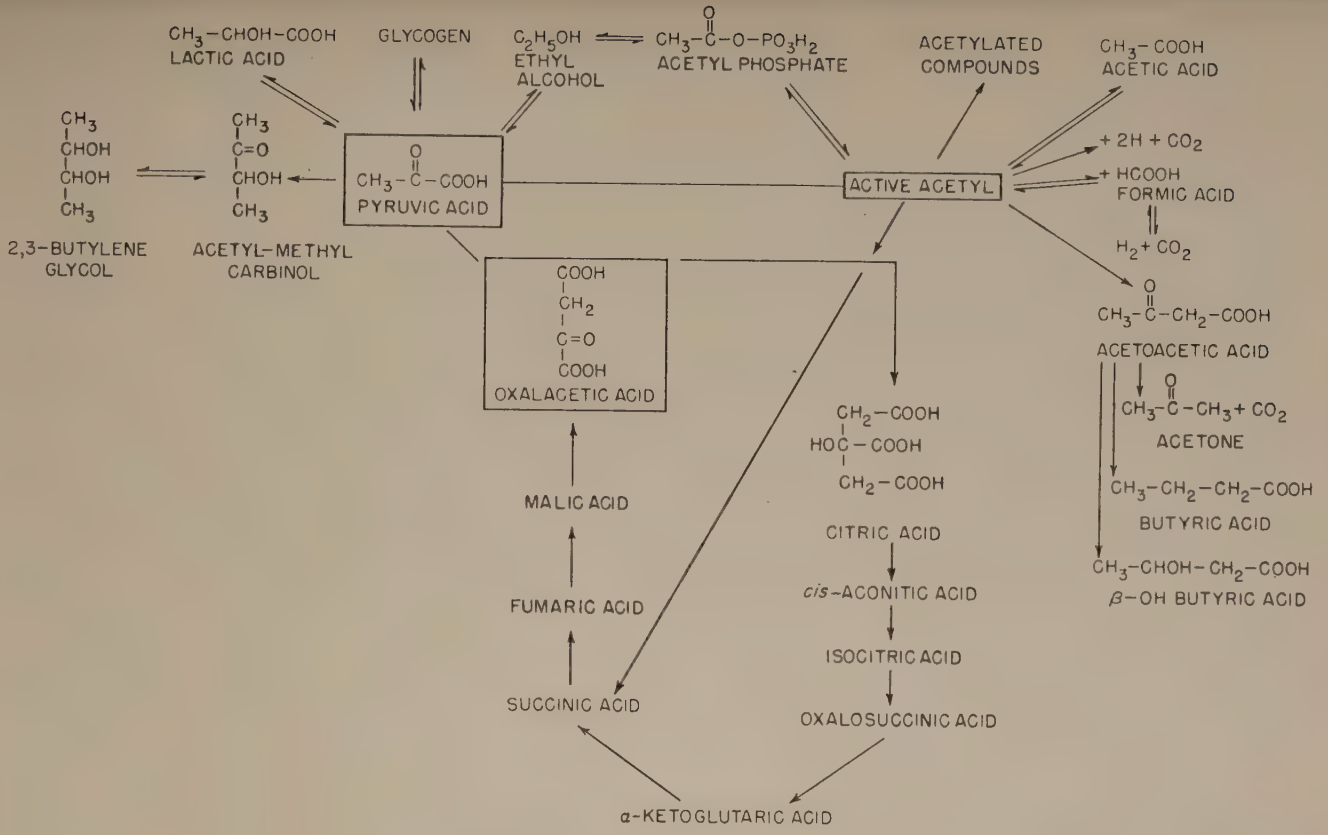

Fig. 1 The reactions of pyruvate.

ide, one further point should be mentioned. It is rare, indeed, to find a tissue or organism which possesses only one reaction by which pyruvate is metabolized. In virtually any tissue there are competing systems and circumstances determining what proportions and what end products may be found. Certain experimental difficulties may be avoided by the preparation of isolated enzyme systems, but in cruder systems and in the living cell itself there is ample evidence that such competition plays an important role. Again, for purposes of simplicity we shall merely point out that such competition exists, and concern ourselves mainly with the separate pathways, recognizing that not all the pyruvate may pass through any one of them.

Certainly the best established and generally accepted respiratory cycle is the citric acid cycle. So well known, so completely proven is this pathway, that any survey of our present knowledge of respiratory cycles is bound to devote most of its attention to this example. But the purpose of this paper is to look beyond our present knowledge and to explore areas where data are fragmentary, and proof is lacking, to see whether there may yet be other pathways in addition to the citric cycle. We shall take it as proven that the citric acid cycle exists, and that the reactions involved are substantially as written in figure 1. It is further granted that the citrate formed is asymmetrical and so are, perhaps, the succinate and fumarate, or some portion of them (perhaps undissociated). And we shall assume further that knowledge of this cycle is so well disseminated that minute description is not necessary. Certainly one in the field of contemporary biochemistry would have detailed knowledge of this cycle. This represents the area of sound information, the consolidated portion of our knowledge of which we may be justly proud.

However, because this cycle is so well established and because most laboratories are in such excellent agreement on the actual facts, it serves admirably as an illustration of the properties of cycles, considered in the abstract. It is perhaps easi-

est to begin by pointing out what a cycle is not, as well as what it is. First, it is not merely a reversible reaction. The shuttle between lactate and pyruvate is not a cycle. Rather, a cycle is characterized by the passage of a given molecule through a series of transformations, each of which alters it in some significant way. These alterations are, of course, normally catalyzed by different enzymes, presumably so located in the cell that the end product of one is in close proximity to the next enzyme which can alter it. At least, study of mitochondria or the granules from bacterial cells gives one this impression of localization although, even here, one is dealing with some degree of disorganization of the process as it may occur in the living cell.

Second, however, a series of reactions of this sort is not yet a cycle, since the Meyerhof-Embden system possesses just these properties and yet we do not consider it a cycle. Rather, the further characteristic of a cycle is that, somewhere in it, one of the products of a component reaction reacts with a substance external to the cycle (or perhaps even a part of it) to yield a further substance identical with or capable of being transformed into an earlier component of the reaction series. In the citric acid cycle, of course, this is the condensation of oxalacetate and acetyl to form citrate. It is this "feedback" mechanism which characterizes cycles and permits their catalytic activity to assume proportions much beyond that of the actual content of the component substances in the tissues. That is, in such cycles, a new parameter is added which unquestionably has far-reaching consequences for living cells. In thinking about how a living cell may employ the mechanisms revealed by enzymatic studies, two factors of flexibility are evident, which are not dependent upon reversibility, even in the purely theoretical sense. These are the feedback mechanism and the competition or alternative pathway, both of which have been previously mentioned. So much for the general properties of the cycles.

Our problem today, however, is to look, if we can, beyond the area of established knowledge to see if we may discern

the presence of other cycles of similar significance to that of the citric acid cycle. We ought first to consider the probabilities that there is more than one such cycle in living cells. One estimates such probabilities, not upon specific data, but upon the general knowledge accumulated by biological science concerning how organisms live. A most fundamental principle derived from this knowledge is that the living cell can adjust, it can adapt to changing environment, and in so doing it can overcome obstacles. Not only can it, but in fact it must. It is the old story of "root hog, or die." Having learned the principle of the feed-back mechanism and its potential uses, is it conceivable that a living cell would not find other uses for it? Is it conceivable that all life is hung on one peg? Having devised, by means beyond our powers to understand, such a sensitive and delicate tool as the citric acid cycle, is it probable that nature would not try again? Indeed, there are such other cycles: the Krebs urea cycle, the proline cycle, the methyl cycle. For these reasons, I think that there is a good probability of other respiratory cycles existing in the living cell. If this be teleology, please note that we are using it only to estimate probability, not as proof.

At the onset, I wish to make one point clear; namely, that the presence of a citric acid cycle in an organism does not exclude the presence of other cycles which may accomplish the same end results. Because it has been demonstrated that most of the enzymes of the citric acid cycle occur in an organism does not thereby say that another respiratory cycle does not exist. There can be 2, 5, or 10; but having shown the presence of one does not thereby exclude the others. There *must* be alternative pathways in varied organisms which may exist side by side and may compete with each other. Further, some organisms are bound to be found in which the citric acid cycle is perhaps undetectable and an alternative is substituted. Witness, indeed, the comparable situation in the *Leuconostoc* where the majority of the Meyerhof-Embden enzymes are present, but where the evidence is quite clear

that an alternative pathway is employed (Gunsalus and Gibbs, '52).

What, then, is the evidence that other respiratory cycles exist in organisms? I shall necessarily deal with two kinds of information; first, the type which provides evidence for an additional cycle; second, the type which provides evidence of occurrences not explainable by the citric acid cycle. This second type of evidence may be considered as indicating either that there are some aspects of the citric acid cycle which we do not yet know or that there are yet other respiratory cycles operating.

Turning to the first type of information, I feel that there is sufficient evidence to establish the existence of a second respiratory cycle. The key reaction which establishes it as a cycle is the condensation of two acetyls to succinate (as shown in fig. 1). The evidence for this reaction is derived mainly from the work of Foster, Carson, and colleagues on *Rhizopus* (Foster et al., '49; Carson et al., '51), and Ajl ('50, '51a, b, c; Ajl and Kamen, '51; Ajl and Wong, '51; Ajl et al., '51) on bacteria. For example, Foster et al. ('49) showed that, in a strain of *Rhizopus nigricans* yielding fumaric acid from ethanol under aerobic conditions, the amount of fumarate formed is such (72% at its highest) as to virtually exclude its origin from citrate. With methyl-labeled ethanol, the tracer was found only in the methylene carbons of the fumarate; with carboxy-labeled ethanol, the label was in only the carboxyl groups. The formation from citrate was excluded, under the conditions employed, by a variety of experimental treatments. In a similar way, lactate formation under aerobic conditions has been shown to involve a $2C_2$ condensation in a similar mold (Carson et al., '51). In *Escherichia coli* grown aerobically with acetate as the principal substrate for growth, Ajl and Kamen ('51) reported that when methyl-labeled acetate was oxidized in the presence of various unlabeled members of the citric acid cycle (from α-ketoglutarate on) there was appreciable accumulation of the label in all substrates except α-ketoglutarate. In comparable experiments

using *Micrococcus lysodeikticus* (Ajl et al., '51) the label was found in the α-ketoglutarate. In *Aerobacter aerogenes* grown with citrate, evidence was presented (Ajl and Wong, '51) for the simultaneous occurrence of the $2C_2$ condensation as well as the citric acid cycle. The arguments are too complex to be described here and the body of data itself must be examined, but it may be paraphrased as follows: with citrate-grown cells, all intermediates contained radioactivity; with acetate-grown cells, only the C_4 dicarboxy acids and pyruvate possessed radioactivity.

It seems to me that in the work of both groups of investigators there is clear evidence that a condensation of $2C_2$ intermediates to succinate occurs. Further, under certain of the experimental conditions employed, most if not all of the C_2 is metabolized by this pathway. The experimental conditions were chosen, indeed, to permit demonstration of this reaction. However these data do not imply, as some have supposed, that under other conditions a citric acid cycle does not occur. The point is that in addition to a citric acid cycle there exists, perhaps in most organisms, a reaction permitting a C_2 intermediate derivable from pyruvate to enter a cyclic process at the stage of succinate, and this reaction may account for a portion of the metabolism of pyruvate. The quantitative importance of this reaction may well vary with organism and cultural or experimental conditions. From indications already in the literature it is reasonably certain that a more positive type of evidence, namely the actual isolation of the enzyme condensing $2C_2$ to succinate, is likely to be available before too long.

Here then are the two known respiratory cycles. It is of importance to note that for both cycles the pathway involves succinate, fumarate, malate, and oxalacetate. These 4 compounds therefore represent not only an area where the two cycles overlap, but also a pathway through which all the carbon of the cell, respired by a cyclic process, must pass. Is this contingency likely, considered from the biological viewpoint? I think not, but experimental evidence of respiratory

cycles not involving these compounds is lacking, and should be sought. If these are not found we have in nature two such indispensable bottlenecks: the pathway from triose phosphate to pyruvate and that from succinate to pyruvate, both of which are readily reversible.

We now come to the second type of information which provides evidence of occurrences not explainable by the citric acid or the "dicarboxy acid" cycle, yet in which there is some evidence of a cyclic process. These occurrences may mean either that there is some aspect of the two established cycles which is not yet known or else that yet another pathway is involved. There is a variety of indications of another pathway which seem, however, always to be based upon the alleged complete absence of the citric or dicarboxy acid cycles. Normally, they are answerable experimentally by subsequent demonstrations that one or both of the other cycles does exist in the organism in question, and the issue is therefore considered closed. We may, however, be missing some evidences of other respiratory cycles by this approach. The question we really wish to ask is, is there any evidence for phenomena not explainable upon the assumption that both of the known cycles are present?

While there may be other cases, I happen to be especially familiar with one. This concerns the reaction of bacteria and animal tissues to streptomycin. While we have, in the past, reported these studies from the viewpoint of the mode of action of streptomycin (Umbreit, '49; Oginsky et al., '49; Umbreit et al., '51), let us, today, simply consider streptomycin as a tool. Let us forget that it is an antibiotic and consider merely that it is an inhibitor of some step in a respiratory cycle. What streptomycin does when properly applied to either bacteria or animal tissue is to prevent the entrance of pyruvate into the respiratory cycle. For example, when bacterial cells are so grown that they do not oxidize added acetate, pyruvate is oxidized to acetate, and then stops. But pyruvate plus oxalacetate are oxidized to completion. When streptomycin is applied, pyruvate and oxalacetate are oxi-

dized, not to completion but to the oxidation state of acetate; and indeed acetate accumulates. This certainly looks as though streptomycin is inhibiting one or more of the steps involved in entering the citric acid cycle. The type of data obtained is shown in table 1. In the absence of streptomycin, succinate, fumarate, malate, and oxalacetate are oxidized toward completion. In the presence of streptomycin, oxidation of all proceeds to the stage of acetate, and there stops.

The known pathways involved in this oxidative process are shown in figure 2. When cells are grown in air, comparable to the conditions used by Ajl ('50), an acetate oxidation system is present. Since this system is not inhibited by strepto-

TABLE 1

Extent of substrate oxidation by E. coli

SUBSTRATE	NORMAL	NORMAL + STREPTOMYCIN	THEORY TO ACETATE
		(Moles O_2/mole substrate)	
Pyruvate	0.44	0.42	0.5
Oxalacetate	> 0.87	0.5	0.5
Malate	> 2.3	0.77	1.0
Fumarate	1.42	1.0	1.0
Pyruvate + oxalacetate	> 0.83	0.56	0.5

mycin, the action of this inhibitor does not carry beyond the point of this special acetate-oxidizing system and we may ignore it henceforth. In cells grown without air in which the acetate-oxidizing system is absent, streptomycin inhibits the oxidation of pyruvate and oxalacetate. One might well assume that inhibition occurs of the reactions pyruvate to acetyl plus oxalacetate to citrate and thus around the cycle, and the problem merely becomes one of which step is inhibited by streptomycin. It is true that such cells do not oxidize added citrate, *cis*-aconitate, and α-ketoglutarate, but this may merely mean that these compounds cannot penetrate through the intact cell surface. This has actually been shown to be the case with α-ketoglutarate. Glutamate is readily oxidized to completion and it can be easily shown that α-keto-

glutarate accumulates in the early stages of the oxidation inside the cell, and later disappears. Hence, such substances as citrate and isocitrate may be inactive because they do not penetrate into the cell. The enzyme which condenses acetyl and oxalacetate is present as well as aconitase. It would seem that the only problem is: at which point in reactions 1, 2, 3, or 4 does streptomycin act? What happens, experimentally, however, is that streptomycin cannot be shown to inhibit any of them. It does not inhibit the conversion of pyruvate to active

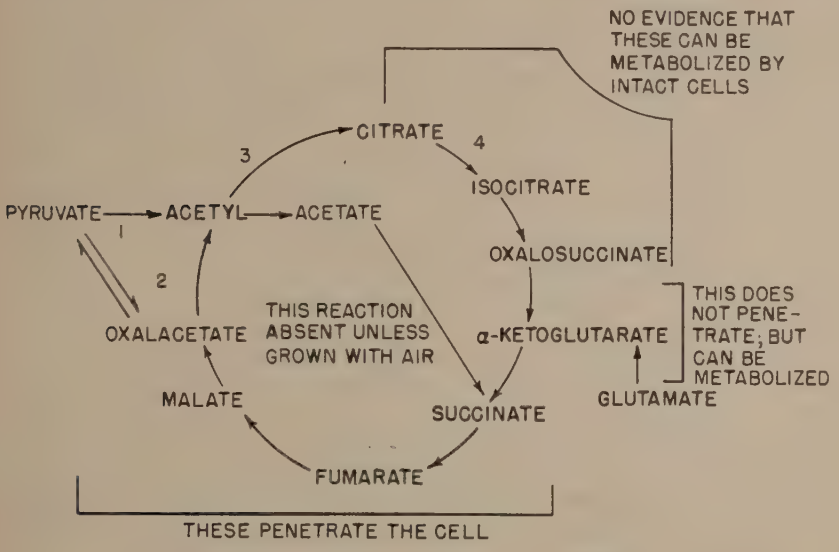

Fig. 2 Respiratory cycles presumed to be present in *E. coli*.

acetyl, or the condensation of this with oxalacetate to form citrate, or the further metabolism of citrate, or indeed the formation of oxalacetate decarboxylase, which is not a necessary reaction when both pyruvate and oxalacetate are added. Much of this information has been published, so rather than review it, let us examine two new kinds of experiments done with rat kidney. The inhibitor, streptomycin, can be made to act on animal tissue, if it is used in relatively high concentrations so that it will penetrate the mitochondria.

Figure 3 presents data on water homogenates of kidney in which there is a marked effect of streptomycin on the oxida-

tion of oxalacetate and pyruvate but no effect upon citrate formation from these substances. One may note that in order to obtain citrate formation one would have to go through active acetyl, hence streptomycin is not inhibiting either the formation of active acetyl or its condensation with oxalacetate to form citrate. It might be acting upon the further metabolism of citrate. The data from isotonic rat kidney homogenates presented in figure 4 show that, while there is a

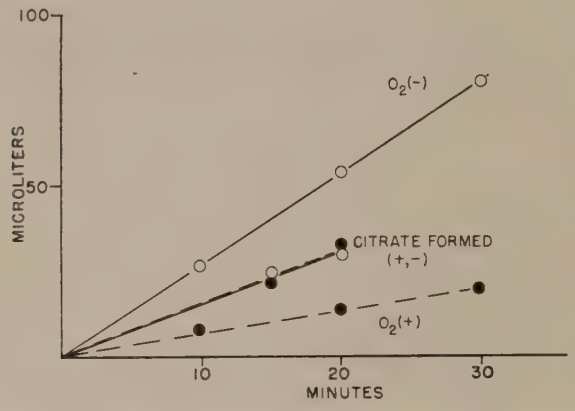

Fig. 3 Oxidation of oxalacetate and pyruvate, and citrate formation by rat kidney water homogenate. Citrate formation: 1.5 ml of 10% rat kidney water homogenate, 0.5 ml of 0.2 *M* oxalacetate, 0.5 ml of 0.1 *M* pyruvate, 0.1 ml of 0.1 *M* magnesium chloride; potassium phosphate buffer (pH 7.4) ± streptomycin to 5 ml total volume. Oxidation: 0.6 ml of homogenate with similar additions to 3 ml total volume. Dashed lines contain 1 mg streptomycin per milliliter.

marked effect of streptomycin upon the oxidation of oxalacetate and pyruvate, there is essentially no effect on the oxidation of citrate. If the streptomycin is inhibiting the oxidation of oxalacetate and pyruvate and is not inhibiting either the formation of citrate or its further metabolism, which is what these experiments demonstrate, there must be another reaction involved with these substances. Almost the converse of this effect has been reported by Paul et al. ('52) in which furacin at 2 μg per milliliter inhibits the formation of citrate in testes but does not inhibit the oxidation of pyruvate or

glucose. We have therefore the case where citrate formation is inhibited but oxidation is not and the case, with streptomycin, where oxidation is inhibited but citrate formation is not. To return to streptomycin, there seem to be two possibilities: first, that there is something in the way this inhibitor acts which is not understood; i.e., perhaps it is converted into an active inhibitor only when oxalacetate and pyruvate are present, possibly after the manner of fluoroacetate; and second, that even in animal tissues, there ex-

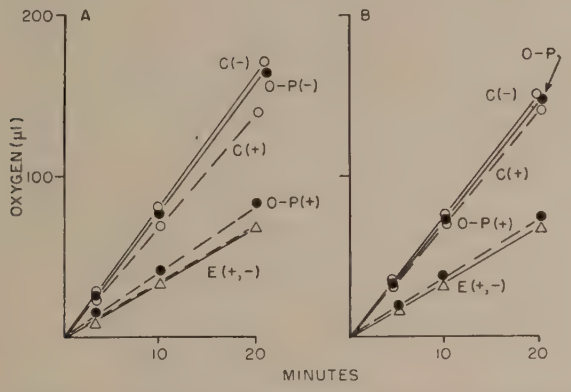

Fig. 4 Oxidation of citrate and oxalacetate-pyruvate by isotonic homogenates of rat kidney. Conditions as described (Umbreit and Tonhazy, '49). O–P = oxalacetate-pyruvate. C = citrate. E = endogenous. A. Direct: 50 µg/ml. B. Incubated: 30µg/ml.

ists another entrance into the terminal respiration system which does not involve condensation to citrate, and that quantitatively this is more important than the pathway through citrate. In short, this may constitute evidence of another respiratory cycle.

But, before we accept it as such, let us carefully examine the first possibility — that streptomycin is converted to an inhibitor in the presence of oxalacetate and pyruvate, not in the presence of citrate, and this inhibitor acts on the further metabolism of citrate. The reaction inhibited would be aconitase. This enzyme was therefore studied.

In this study we employed the spectrophotometric method of Racker ('50) using either citrate as the substrate and measuring the increase in absorption at 2400 A or more commonly, using *cis*-aconitate as the substrate and measuring the loss of absorption at 2400 A as *cis*-aconitate was converted to citrate and isocitrate. We initially had a reasonable amount of difficulty in measuring this enzyme due to its relative instability at very high dilution, particularly since we did not wish to employ stabilizing agents but wanted the enzyme in

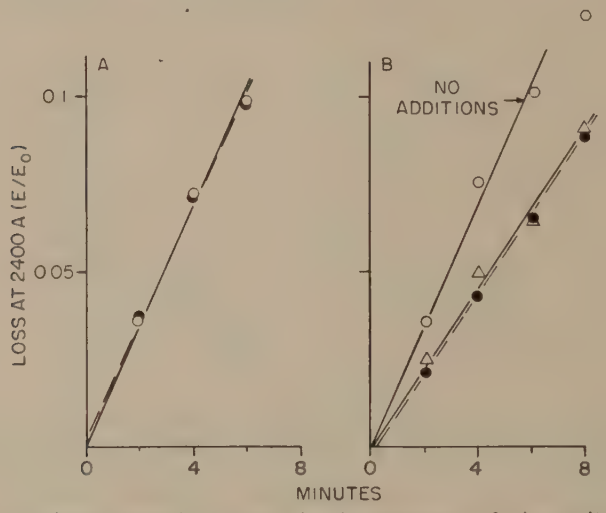

Fig. 5 Aconitase. Reaction plotted is disappearance of *cis*-aconitate. Dashed lines represent streptomycin additions.

its most sensitive state. We finally resorted to dilution in purified gelatin and under these circumstances were able to obtain consistent measurements of the aconitase from heart and kidney over periods as long as 10 minutes. We found no evidence of streptomycin inhibition nor any positive evidence of the formation, from streptomycin, of a product toxic to this enzyme.

Typical data are shown in figure 5 for an aconitase preparation from rat heart in which the reaction, *cis*-aconitate to citrate, is followed by loss of absorption at 2400 A. The substrate concentration is only 6×10^{-5} *M*. First, from part A

it may be noted that very high concentrations of streptomycin (500 μg/ml) had no effect upon aconitase. In part B a different treatment was employed. A suspension of *E. coli* was incubated in the presence of oxalacetate and pyruvate with and without streptomycin. At the end of a two-hour incubation period, at which time there was a marked effect in the bacteria on the oxidation of oxalacetate and pyruvate, the reaction was stopped by heat and the solution from which the heat-killed cells had been removed was applied to the aconitase system. The amount of streptomycin supplied to the bacteria was such that the fraction of the extract placed in contact with the aconitase was equivalent to 20 μg of streptomycin. This amount was adequate to show discernible inhibition in the oxidation of oxalacetate and pyruvate when these were oxidized by animal homogenates (Umbreit and Tonhazy, '49). Therefore one might presume that if some toxic product other than streptomycin itself were formed by the oxidation of oxalacetate and pyruvate, at least some inhibition should be observed, yet none was found. There was some inhibition of aconitase by the *E. coli* extracts, but no further inhibition due to the presence of streptomycin.

The type of data presented showing that streptomycin has no effect upon any of the known steps in that pathway via citrate formation or oxidation in the respiratory cycle while yet having a marked effect upon the oxidation of oxalacetate and pyruvate, is not, and possibly never can be, conclusive. From the very nature of the logic employed one cannot conclude that streptomycin does not act, by some means as yet not tested, in this pathway. But one can say that no positive evidence has been found for its action in this manner, that the experiments have been as adequate as we can make them, and that in all experiments there is not even a hint of inhibition. One might debate this issue for hours, but a definitive conclusion is impossible. Nevertheless, at our present state of information the data cannot be explained upon the assumption of the citric acid cycle, even granting that there is adequate proof that this cycle exists in the systems with

which we are working. It is important, therefore, to examine the possibility that, in addition to a citric acid cycle, there is still another respiratory cycle.

Figure 6 shows some alternative possibilities based upon the possible occurrence of a $C_3 + C_4$ condensation, the most

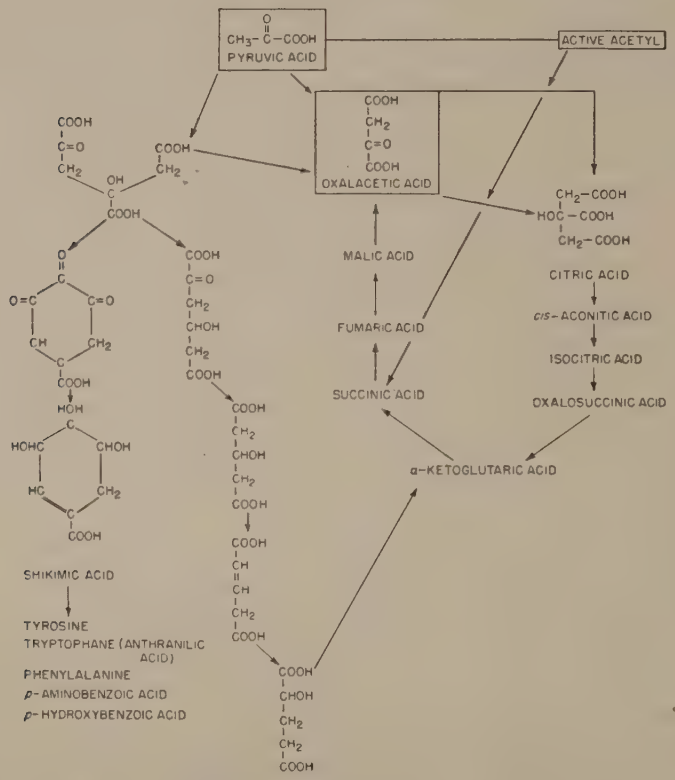

Fig. 6 Alternative possibilities of respiratory cycles.

likely intermediate of which might be citromalic acid. This compound could conceivably follow one or more of three pathways. First, it might be decarboxylated to citrate. Second, it might be decarboxylated at the center carboxyl to form intermediates which could be transformed to α-ketoglutaric acid. Third, it might possibly be reduced, rather than oxidized, and the ring closed to form what one might assume to be a pre-

cursor of shikimic acid and thus be a precursor of ring structures.

Martius ('43) prepared this citromalic acid in the form of a lactone, but concluded that it was not active metabolically. His published experiments revealed that citromalic acid did not form citrate under the conditions of an experiment in which acetate + oxalacetate did yield citrate. However, if we are to consider the possibility of decarboxylation of the center carboxyl or conversion to shikimic acid, we would naturally not expect citrate as an intermediate. It therefore became necessary to re-examine citromalic acid from this point of view. However, the material is not rapidly metabolized in either animal or bacteria tissues, and is relatively inert.

With respect to the possibility of shikimic acid or a related substance acting as an intermediate in these reactions, one might first suppose that if adequate shikimic acid were supplied, growth would not be inhibited by streptomycin. This does not happen; shikimic acid does not antagonize streptomycin. Further, both the shikimic-accumulating and the shikimic-requiring strains of *E. coli* possess the oxalacetate-pyruvate reaction and in both cases it is sensitive to streptomycin. It is true that shikimic acid is oxidized by *E. coli,* but our impression is that it is oxidized via the pathways associated with the oxidation of ring structures.

As you know, there is a report (Rapoport and Wagner, '51) that a 7-carbon phosphorylated compound, 2-phospho-4-hydroxy-4-carboxy adipic acid, occurs in dog liver. Its structure is illustrated in figure 7. For convenience, without further claims of structure, we shall call it the Rapoport compound. The first problem was to determine whether this compound did indeed exist, and whether it was an active intermediate. The Rapoport compound is characterized by the fact that its mercury salt and its barium salt are insoluble, yet the compound is stable to acid hydrolysis. A fractionation scheme was therefore devised based on these properties, as shown in figure 8.

In essence, the fractionation procedure consists in isolating the fraction containing the mercury salt of the Rapoport compound, which fraction will also include adenylic acid, adenosinetriphosphate (ATP), and adenosinediphosphate (ADP), as well as certain other nucleotides and coenzymes. From this fraction the barium salt is prepared, thus eliminating the adenylic acid. After removal of barium, the supernatant material is hydrolyzed with acid (to which the Rapoport compound is stable) thus converting ATP and ADP to adenylic acid. The barium salt is again prepared, and the barium fraction now contains the Rapoport compound plus certain impurities arising from the coenzymes and perhaps other compounds. It was

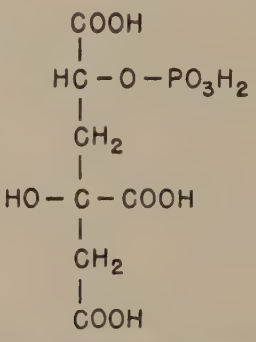

Fig. 7 Supposed structure of Rapoport compound.

found that this latter barium salt, in contrast to most known barium salts, was mostly insoluble in normal acetic acid, so that a further separation was made into two fractions, one acetic acid soluble, termed RS and the other acetic acid insoluble, termed RI.

Before examining, or indeed obtaining, evidence as to the exact nature of the compound or compounds present in these two fractions, it was of interest to determine whether they were metabolically active. For this purpose, Clark of our Isotopes Department injected radioactive phosphorus into several rats, whose livers were then fractionated according to this scheme. Data obtained on the specific activities of the fractions in question are given in table 2, from which it is apparent that

the acetic acid-insoluble fraction (RI) is quite radioactive and its activity is higher than the ester phosphate which in turn is higher than that of the nucleic acids. There is further evidence that the RI fraction contains a metabolically active intermediate since, as the time after injection is prolonged, the specific activity drops to that resembling the ester phosphate.

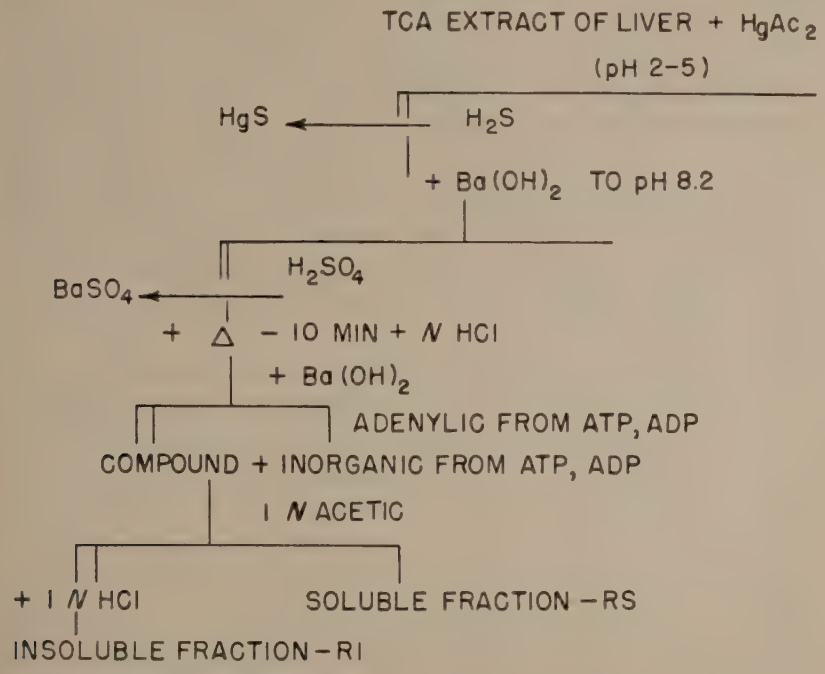

Fig. 8 Fractionation scheme for isolation of Rapoport compound.

TABLE 2

Incorporation of radioactive phosphorus

	SPECIFIC ACTIVITY AT INDICATED HOURS AFTER P^{32} INJECTION		
	4	6	24
Rapoport soluble	3.8	0	0.67
Rapoport insoluble	188.0	21.4	2.1
Ester phosphorus	. . .	10.5	1.24
Nucleic acid phosphorus	2.68	2.2	.

It is, I hope, recognized that these studies are in a most preliminary stage and all of the various types of evidence that are obtainable have not yet been undertaken. There is, I think, no question but that RI contains an active phosphorylated intermediate, but the identity of this intermediate is as yet unknown. The described properties of the Rapoport compound are as yet very few and even proof of structure is not rigorously established. One property available at this time for more or less qualitative characterization is its curious reaction with Nessler's reagent, in which it gives a color with a maximum absorption at 383 mμ. We find that fraction RI gives a very intense color with Nessler's reagent which is not due to ammonia and which is roughly proportional to the phosphorus content. There is, therefore, presumptive evidence that RI contains the Rapoport compound but at what state of purity or whether the radioactivity is indeed associated with it is not known as yet.

We therefore feel that, while proof of an alternative path to entrance into the terminal respiration systems not involving the known cycles is by no means available, now is not the time to abandon search for such pathways and there are indications, from the action of streptomycin and from the phosphorylation studies, that such alternatives do exist. This is admittedly a penetration beyond what is at present known for certain, but that was the purpose of this paper. Let us not fall into the easy path which regards such indications as "not proven" and therefore impossible. Let us make use of these indications to find out whether there are more respiratory cycles than those for which we now have proof. To borrow from Hamlet, there may be more things in Heaven and Earth than are yet dreamed of in our poor philosophy.

DISCUSSION

Chairman Carson: In connection with the Rapoport compound, I should like to ask whether chromatographic purity has been established for this compound? And is it not easily handled?

UMBREIT: The purity has not been established; it is quite easily handled. It moves very rapidly. Our difficulty has been that we have not had enough of the radioactive material to make sure that all the radioactivity is associated with the one spot that we do get. Isolating larger quantities from horse liver without the radioactivity, we do find only one spot; but our methods of developing these spots are not too good and we are not at all sure that small amounts of other materials in other places on the chromatogram would show up.

KRAMPITZ: I do not think anyone would disagree with Doctor Umbreit that there are possibilities for the existence of more than one metabolic cycle to account for the process of terminal respiration. We must keep alerted to this fact. On the other hand, before one concludes that several cycles are involved, the data should be much more conclusive than those which are available at the present time. This applies particularly, it seems to me, to the dicarboxylic acid cycle in which the direct oxidative condensation of two acetates to succinate is a cardinal step.

A few years ago Carson and Foster, working with *Rhizopus nigricans,* presented data which strongly indicated that the dicarboxylic acid cycle occurred in this organism. However, in later experiments with the same organism, they showed that the β position of lactate became highly labeled when labeled formate was added to the metabolic mixture. Known pathways of lactate metabolism can explain the type of labeling which they obtained in their earlier work. In view of these results the earlier work cannot be rigorously interpreted as conclusive evidence for the oxidative condensation of two acetates to succinate.

Recently Ajl and co-workers have interpreted evidence obtained from "carrier type" experiments that the dicarboxylic acid cycle and the oxidative condensation of two acetates to succinate occurs in *Escherichia coli.* Resting cells of the organism were allowed to metabolize isotopic acetate in the presence of suspected unlabeled intermediates. In this case, isotopic acetate was trapped to a high degree in succinate but

to a very small degree in α-ketoglutarate. Therefore it was concluded that the dicarboxylic acid cycle was of quantitative importance in *E. coli* and the tricarboxylic of very little, if any, importance.

Mister Swim and I have been concerned with this problem in *E. coli* (acetate adapted) and have also found that carrier α-ketoglutarate does not trap acetate carbon but that succinate does. These results, we feel, are entirely due to the inability of metabolic α-ketoglutarate to equilibrate with the carrier pool and are therefore uninterpretable as far as the mechanism of oxidation of acetate is concerned.

We also have good evidence that the direct oxidative condensation of two acetates to succinate does not occur in *E. coli*. Acetate-1-C^{14} was oxidized anaerobically by *E. coli* with fumarate as the electron acceptor. The stoichiometry of the reaction is

$$1 \text{ acetate} + 4 \text{ fumarate} + H_2O \rightarrow 2\ CO_2 + 4 \text{ succinate.}$$

At the end of the experiment all the isotope was found in the succinate and none in the respiratory carbon dioxide. These results are in agreement with the tricarboxylic acid cycle, but not the dicarboxylic acid cycle. When excess fumarate was added, none of the isotope was found in the residual fumarate, indicating that under these conditions there was no recycling of the succinate. This being the case, using acetate-2-C^{13}, one can more critically evaluate the hypothesis of acetate condensation to succinate. The C^{13} acetate was anaerobically oxidized by *E. coli* in the presence of unlabeled fumarate. The isotopic succinate was isolated and degraded so as to obtain the two methylene groups as ethylene and the two carboxyl groups as carbon dioxide. The carbon dioxide contained no isotope. By determining the mass of the ethylene (methylene groups of succinate) one can determine if the direct oxidative condensation of two acetates to succinate occurred, or if one acetate entered the succinate oxidatively by way of the tricarboxylic acid cycle. In the former case, the ethylene would have a mass of 30 and in the latter its mass would be 29. Acetate containing C^{12} would, of course, give rise to ethylene of mass 28. Mass

ratios of the ethylene species present indicated no ethylene of mass 30, whereas the 29 to 28 mass ratio showed that approximately 25% of the succinate was derived by entrance of one molecule of acetate into the succinate molecule. These data support the tricarboxylic acid cycle in *E. coli,* and lend no support to the direct oxidative condensation of two acetates to succinate.

The inability of whole cells of *E. coli* to oxidize citrate is no longer an objection to the tricarboxylic acid cycle, since Lara and Stokes have recently shown that by special treatment of the cells a very active oxidation can be demonstrated. I believe that caution should be exercised in interpreting fragmentary data regarding the existences of various cycles in the process of terminal respiration.

CARSON: It seems as if the evidence concerning this particular system is convincing, and the dicarboxylic acid cycle does not function there.

KAPLAN: Doctor Umbreit, do you mean that the point of action of streptomycin is on citrate utilization, or that one gets citrate formation with no pyruvate oxidation, or that there is incomplete oxidation of pyruvate?

UMBREIT: The point I am trying to make is that streptomycin will prevent, in general terms, the entrance of pyruvate into the terminal respiration system. If we follow the known reactions for getting into the citric acid cycle, we do not find any of the reactions inhibited by streptomycin, yet the over-all oxidation of pyruvate plus oxalacetate is inhibited. This is not theoretical; it is what the experimental data show.

KAPLAN: You do not get any citrate accumulated?

UMBREIT: No. And further we do not get any inhibition of citrate oxidation nor any inhibition of citrate formation.

KAPLAN: What happened to the pyruvate?

UMBREIT: I do not exactly understand what you are trying to get at.

KAPLAN: I mean if you get no citrate accumulation and no oxidation, the pyruvates just do not move.

UMBREIT: I think it may well be that in the streptomycin-inhibited system, the pyruvate just does not move, that is, in the animal systems we have been dealing with here. In bacteria, there is the system taking pyruvate to acetate, which is not influenced by streptomycin; and the pyruvate will always end up at the oxidation state of acetate. You can actually isolate the acetate, but in the animal systems, such as in the kidney homogenate which we have mentioned, the pyruvate probably just remains there.

LAMPEN: Maybe this bears on this same point. I was wondering about the actual values that you obtained on these graphs. From the ordinates of these curves it looked as though the oxygen consumption in the system was about the same as the citrate formation. Were these in terms of micromoles?

UMBREIT: These are in terms of microliters in that particular illustration.

LAMPEN: So there is a large formation of citrate.

UMBREIT: You see, in a water homogenate citrate will tend to pile up, which is the reason that a water homogenate was used in that case.

LAMPEN: From your illustrations I would conclude that the major product in the pyruvate-oxalacetate system was citrate. Is that correct?

UMBREIT: Yes, I think that is probably true, and is due to the way the system was set up.

LAMPEN: And yet there is no effect of streptomycin on citrate accumulation? Is that in agreement with the idea that the pyruvate and oxalacetate are going through to a different product?

UMBREIT: Yes. If we take the oxidation values, the oxidation of the streptomycin-inhibited system would be adequate to account for the citrate formed. The oxidation of the oxalacetate plus pyruvate in the system which was not streptomycin inhibited is greater in amount than that which would be accounted for by the citrate formed. That is, there was another system present capable of oxidizing these two compounds and this was the system that was cut out by the

streptomycin. But streptomycin had no effect on the citrate formation. There were two systems operating simultaneously.

LAMPEN: That other system, then, is a minor system as you have it set up.

UMBREIT: Yes, in the water homogenate from animal tissues it would be relatively low. The purpose of this kind of data is to try to build up the citrate-forming system as much as possible in order to determine whether there is streptomycin inhibition. There is none, and I would be perfectly happy if streptomycin would act at any of these points; it just happens that it does not.

LICHSTEIN: I should like to ask a question concerning the mode of action of streptomycin rather than its use as a tool in the study of terminal respiration. We have obtained presumptive evidence with *Saccharomyces fragilis* that this antibiotic inhibits the synthesis of pantothenic acid or coenzyme A. Have you any information from your enzyme studies that streptomycin inhibition may be reversed by pantothenate or coenzyme A?

UMBREIT: No, we have been unable to do any reversal of streptomycin inhibition, with pantothenate, coenzyme A, or anything else. Once streptomycin has united with the enzyme, it is irreversibly inhibited. However, if the resting-cell suspension is inhibited with streptomycin, and the organisms placed into a fresh medium, growth will frequently be obtained. This appears to be due to formation of more of the necessary enzyme, whatever it is, rather than to the dissociation of streptomycin from the inhibited enzyme. However, we have been unable to grow the sensitive cell in a medium containing sufficient streptomycin to inhibit it in its normal state by any additions other than certain agents which will react to the streptomycin itself and destroy it.

H. G. WOOD: I want to point out that use of tracers may lead to erroneous conclusions; this is especially true when carriers are added to trap the labeled intermediates that may be formed. Recently there have been numerous papers which present evidence that there is an oxidative shunt

through acetate condensation to succinate rather than oxidation via the citric acid cycle. While there is a fair probability that such a cycle will be established, the evidence with tracers is by no means conclusive. In these tracer experiments, C^{14}-acetate and unlabeled succinate, α-ketoglutarate, etc., have been added in order to detect the occurrence of intermediates of the oxidative cycle. The chief concern in this use of isotopes is whether the compound added as a trapping agent is in equilibrium with the metabolic pools. In reality, this method can be considered reasonably reliable only if the material is cleared from inside the cell and the identity of the compound that is occurring in the cycle is known.

I think that Doctor Krampitz did not mention the experience that his group, Saz and Swim in particular, have had in carrier experiments. They have used C^{14}-acetate with *E. coli*. In their studies both the external carrier α-ketoglutarate, succinate, etc., and the internal α-ketoglutarate and succinate, as obtained by extraction from a large mass of cells, have been analyzed for C^{14}. The two pools were entirely different insofar as their isotope content was concerned. The α-ketoglutarate, succinate, etc., from inside the cell were completely in equilibrium with the C^{14}-acetate, whereas the external α-ketoglutarate had low activity. Obviously the external α-ketoglutarate is not a reliable indicator of the internal activity of the cell, and exclusion of a cycle on the basis of the radioactivity of the external compounds is not warranted. The important thing to bear in mind in doing tracer studies is that one must actually get out the material that is turning over. The use of cell fragments does not remove the difficulty; Saz obtained similar nonequilibration with lysed preparation of *Micrococcus lysodeikticus*. In his experiments the cell fragments were extracted.

Even if the experiments are done with extracts, unless one knows that the added compound is the true intermediate in the particular reaction, and that it is not a derivative of CoA, for example, one cannot exclude the role of a particular cycle. Extreme care must be exercised in interpreting

carrier-trapping experiments, even those where cell-free extracts are used.

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STUDIES CONCERNING THE STRUCTURE OF COENZYME A

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FIVE FIGURES

Since much of this program will be concerned with the participation of coenzyme A in a variety of transacetylation reactions, I feel that a discussion of our present knowledge of the structure of the coenzyme will give us a better background for the understanding of its metabolic function.

In conjunction with the work on the isolation and metabolic function of CoA during the past 5 years in Doctor Lipmann's laboratory, an investigation into the structure of the coenzyme was conducted. Until very recently, only concentrates of the coenzyme ranging in purity from 10–60% were available for this study. Therefore, much of the information on the various linkages in the coenzyme was obtained on impure samples by means of analysis of specific enzymatic degradation products (Novelli et al., '50). Recently samples of CoA better than 90% pure have become available (Gregory et al., '52) and most of the earlier observations have been confirmed with this substance.

The analysis of one of the best preparations of CoA is shown in table 1, which gives the molecular composition of the coenzyme.

These data suggest that the compound consists of 1 mole each of pantothenate, adenine, ribose, and β-mercaptoethylamine, and 3 moles of phosphate. If these molecules are strung together with the elimination of water, a calculated

molecular weight of 767 is obtained. This is in rather good agreement with the value of 800 ± 50 (Novelli et al., '49) which was obtained on much less pure material by the Northrup diffusion method.

These data, together with information on the enzymatic hydrolysis, suggest the structural formula given in figure 1.

TABLE 1

Molecular composition of coenzyme A [a]

	CALCULATED	FOUND	RATIO
	%	%	
Pantothenic acid	28.6	26.8	1
Adenine	17.6	17.0	1.05
Ribose	19.5	..	..
Phosphorus (total)	12.12	10.6	2.83
Monoester phosphorus	4.04	3.6	0.96
Sulfur	4.18	4.13	1.07
β-Mercaptoethylamine	9.9	..	..

[a] From Gregory, Novelli, and Lipmann ('52).

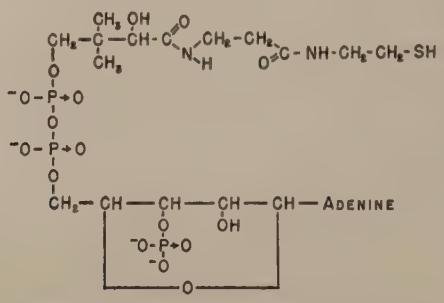

Fig. 1 Provisional structure of CoA.

In this formulation we depict the coenzyme as a dinucleotide similar to triphosphopyridinenucleotide (TPN). Adenosine is connected to pantothenate through a pyrophosphate bridge, and β-mercaptoethylamine is connected to pantothenate through the carboxyl group of the β-alanine portion. The evidence which has led to this particular formulation will be considered in detail as follows.

PANTOTHENIC ACID

CoA will not substitute for the vitamin for *Lactobacillus arabinosus,* the usual test organism for pantothenic acid (PA) (Lipmann et al., '47). As a matter of fact, the presence of PA in the coenzyme was almost missed because of this fact. It was only after Guirard, working in Williams' laboratory in Texas, showed a large content of β-alanine (by acid hydrolysis) that the presence of PA in the coenzyme was suspected. After considerable trial and error it was eventually found that the PA could be liberated from CoA by the action of two enzymes (Lipmann et al., '47). This is

TABLE 2

Pantothenate liberated from coenzyme A by various treatments [a]

TREATMENT	PANTOTHENATE LIBERATED
	%
None	0
Clarase + papain	0.15
Alkaline phosphatase	1.4
Liver enzyme	4.8
Liver enxyme + sodium fluoride	0.5
Liver enzyme + alkaline phosphatase	9.0
Acid hydrolysis (*β*-alanine)	10.1

[a] Taken from Lipmann et al. ('47).

shown in table 2. Here we see that a mixture of clarase and papain (enzymes which had been used routinely for the liberation of PA from its bound form) had little effect on CoA. Alkaline phosphatase alone, or liver enzyme alone, was likewise unsuccessful. But when these two enzymes were combined, the PA values obtained were almost equivalent to those calculated from the β-alanine content after acid hydrolysis. When this method was applied to a large series of CoA samples of varying degrees of purity, a constant ratio of PA content to coenzyme activity, irrespective of purity, was obtained. These data are presented in table 3. The average value of μg of PA per unit of activity is approximately 0.7. However, the PA values obtained from the β-alanine content

are about 10% higher than the directly determined values. We can assume, therefore, that the correct value of μg of PA per unit lies between 0.7 and 0.8. This value can be used to calculate the unit activity per μ*M* of CoA. Using the factors 0.7 and 0.8, the unit activity per μ*M* would be between 275 and 310, or approximately 300. Calculated with the molecular weight of 767, the PA content of 1 mg of pure CoA would be 1.3 μ*M;* from the value of 300 units per μ*M*, the unit activity of pure CoA per mg would be approximately 390. Assuming the 0.75 μg of PA per unit, pure CoA would contain about 29.3% PA. This value, calculated from unit activity, is remarkably close to the value of 28.6% PA as calculated from the molecular composition (see table 1).

TABLE 3 [a]

Ratio of pantothenate to unit activity of a variety of coenzyme A samples

PREP. NO.	ACTIVITY	PA FROM β-ALANINE AFTER ACID HYDROLYSIS	PA, DIRECT ASSAY AFTER ENZYME TREATMENT	μG OF PA: UNIT ACTIVITY
	Units/mg	%	%	
105	9	1.1	0.7	0.78
100	10	1.0	1.0	1.00
72	26	2.0	1.8	0.69
101	32	2.0	1.8	0.56
102	60	4.2	3.7	0.62
99	65	4.7	4.4	0.68
103	100	6.5	6.5	0.65
A′	130	10.0	9.3	0.72
A	132	9.9	8.4	0.64
25–DeV–22	150	..	11.0	0.73
127–DeV–22	170	..	13.1	0.78
C 50a	210	..	14.9	0.71
182d	357	..	25.0	0.70
140a	370	..	22.2	0.60
146b	387	..	26.8	0.67

[a] Modified from Lipmann et al. ('47).

From these observations we can already make some inferences concerning CoA structure.

1. It is clear that PA is doubly bound, since neither enzyme alone could liberate the vitamin.

2. Since the alkaline phosphatase used in these experiments was a highly purified sample (Schmidt), it is reasonable to suppose that one of the linkages to PA is through a phosphate group, especially since the release of PA is accompanied by the liberation of phosphate.
3. This preparation of alkaline phosphatase has both di- and monoesterase activity, i.e., it can split phosphate from nucleic acid, diphosphopyridinenucleotide (DPN), and diphenylphosphate. Crude preparations of clarase, papain, or mylase-P, which have powerful monoesterase activity, cannot replace alkaline phosphatase in liberating PA. This fact suggests that the phosphate group is doubly bound.

From these experiments, nothing can be said of the linkage being attacked by pigeon liver extract, except that it is not a phosphate linkage. The nature of this cleavage and of the fragment liberated was undisclosed for several years before it was clarified in an unexpected manner. The presence of a rather large amount of sulfur in CoA was recognized by Lipmann et al. ('47, '50) when the first concentrates of CoA were prepared. This material gave both chemical and microbiological tests for sulfur. Gregory, in our laboratory, in studying the sulfur-containing compound, was able to show by paper chromatography of acid hydrolyzates of CoA that the material was not cysteine but was closely related to cysteine in that it contained —SH and gave a ninhydrin spot. At this time Snell and his co-workers were studying a growth factor (Williams, Hoff-Jørgensen, Snell, '49) for *Lactobacillus bulgaricus* (called "LBF") which was able to replace the requirement for pantothenic acid (Brown et al., '50; McRorie et al., '50). They were able to show that LBF was derivable from CoA by alkaline phosphatase treatment, and that, presumably, it is a degradation product of CoA. They (Snell et al., '50) were also able to show, in a very nice study, that LBF is the peptide of PA with β-mercaptoethylamine. Therefore, the sulfur-containing compound

in CoA could be this amine (which is decarboxylated cysteine). These observations were confirmed by Gregory and Lipmann ('52).

Figure 2 is a chromatogram of hydrolyzates of CoA and pantethine along with β-mercaptoethylamine, cysteine, and a mixed disulfide. The chromatogram was developed with ninhydrin; and illustrates the fact that the ninhydrin-reactive material derived from CoA is identical with β-mercaptoethyl-

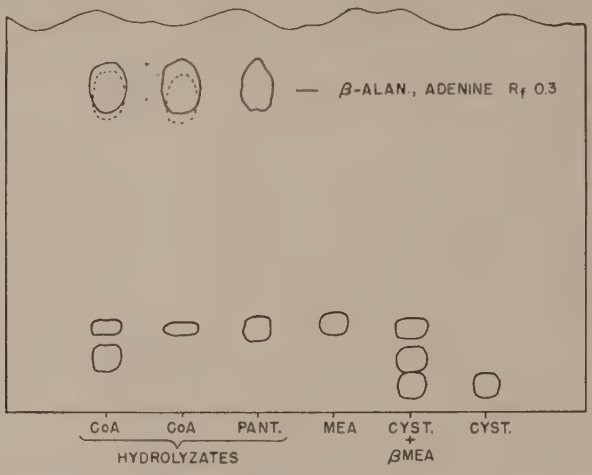

Fig. 2 Chromatogram of hydrolyzates of CoA compared with pantethine and β-mercaptoethylamine (Gregory and Lipmann, '52).

Solvent — sec-Butanol, formic acid; paper — Whatman no. 3.

Abbreviations: PANT., pantethine; βMEA, β-mercaptoethylamine; CYST, Cysteine.

amine and the amine derived from synthetic pantethine. The first CoA spot and the mixture of cysteine and β-mercaptoethylamine illustrate the mixed disulfide obtainable from a crude CoA sample containing cysteine. These data make it clear that the mercaptoamine from CoA is identical with β-mercaptoethylamine. Thus the action of the pigeon liver enzyme is to cleave the linkage between β-mercaptoethylamine and pantothenic acid. In our laboratory Levintow has recently demonstrated the presence of this enzyme in hog kidney.

ADENINE AND PHOSPHATE

In the earliest analysis of CoA, while the material was still only 25% pure (Lipmann et al., '47), the data indicated 2 moles of adenine (determined by the ultraviolet absorption at 260 mμ and characterized as the picrate) and 6 moles of phosphate per mole of PA. As purification progressed, the ratio of adenine to phosphate content decreased and leveled off at 1:3 (Novelli et al., '51). Table 4 gives representative data showing these ratios.

Adenine. As previously mentioned, the presence of adenine was suspected from ultraviolet absorption, and adenine was

TABLE 4

Phosphate, pantothenate, and adenine ratios

CoA SAMPLE	ACTIVITY	MOLAR RATIO	
		Phosphate: PA	Adenine: PA
	Units/mg		
VIII C 23 + 24	88	3.06	1.14
149–MEB–5	170	3.6	1.72
C 43a	210	3.0	1.45
C 83	214	3.85	1.23
VIII C 25 + 26	216	3.29	1.02
C 98 II	217	3.34	1.32
C–123	275	3.15	1.20
115d	305	2.97	0.91

isolated as the picrate. The adenine was further shown to be present as some form of bound adenosine. This was demonstrated by the fact that the adenine calculated from ultraviolet absorption at 260 mμ could be entirely accounted for as adenosine, as measured with adenosine deaminase after dephosphorylation with alkaline phosphatase. We shall consider the nature of this bound adenosine somewhat later.

Phosphate. Of the 3 moles of phosphate present, 1 mole is present as a phosphomonoester while the other two are present as diesters. Consider the data of figure 3. If CoA is treated with an acid phosphatase isolated from the prostate gland, which has only monoesterase activity, only about 35%,

or 1 mole of phosphate, is liberated; whereas, with alkaline phosphatase, which, as pointed out earlier, has both mono- and diesterase activity, all of the phosphate is liberated. From this, one can conclude that one of the phosphate groups is a free monoester, while the remaining phosphates are doubly bound.

The activity curves in figure 3 warrant a brief explanation. During the isolation of CoA it was discovered that the assay system for CoA, the acetylation of sulfanilamide by pigeon liver extract, was able to respond to certain degradation

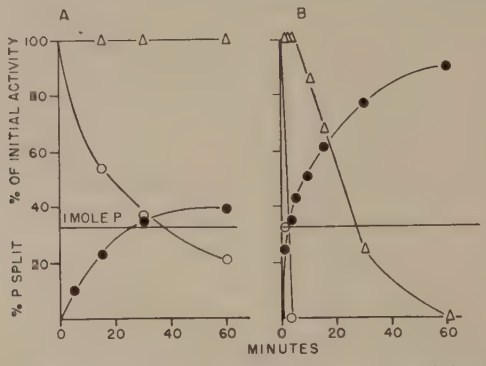

Fig. 3 Time curves of phosphate split and loss of activity by enzymatic hydrolysis of CoA.

△ = Liver assay ○ = Arsenolysis ● = P split

A. Prostate phosphatase (acid). B. Intestinal phosphatase (alkaline).

products of the coenzyme (Novelli et al., '50). If the pigeon liver acetylating system is fractionated with ammonium sulfate between 0.4–0.7 saturation, the acetylating ability is maintained intact. This so-called "purified" assay no longer responds to the split products of CoA. This observation led to the suggestion that the CoA fragments were being resynthesized during the crude assay due to the presence of adenosinetriphosphate (ATP) and the appropriate enzymes (see table 6). With the discovery that the transacetylase reaction, which will be discussed here by Doctor Stadtman, was CoA dependent, an assay for CoA was developed which utilized the arsenolytic decomposition of acetyl phosphate (Stadtman et

al., '51). Since this system is devoid of ATP, it was found to react only with intact CoA.

When CoA is treated with prostate phosphatase and subsequently assayed with the crude liver assay, no change is registered, indicating a replacement of this phosphate group by ATP. The arsenolysis assay, however, shows a rapid loss in activity, but with alkaline phosphatase-treated CoA, *both* assays show a complete loss in activity, although the loss first appears in this liver assay only after approximately 40% of the phosphate is removed. Since the monoesterase activity in alkaline phosphatase is much more potent than the diesterase activity, these data are interpreted as a removal of the monoester group before the attack on the diester.

The data so far presented are similar to those that would be obtained with TPN under similar conditions, and therefore suggest that the doubly-bound phosphate groups exist as a pyrophosphate bridge.

PYROPHOSPHATE BRIDGE

Although the presence of a pyrophosphate bridge in CoA has not been established with absolute certainty, there are a number of observations which lend strong support to the existence of such a structure (Novelli, '51). One of the first observations suggesting the presence of a pyro bridge in CoA was the observation that a combination of prostate phosphatase and a potato extract could replace alkaline phosphatase in the liberation of PA (Novelli et al., '50). This combination caused the complete inactivation of CoA for the test system. Neither enzyme alone was effective in this respect. When this effect was studied more closely, it was found to depend on the order of addition of the enzymes (table 5).

Here it can be seen that *only* when the potato enzyme was allowed to act first was the coenzyme inactivated and phosphate liberated. This observation is interpreted as follows: The potato enzyme splits a linkage, presumably a pyrophosphate bridge, which converts the phosphate groups to

monoesters. Then the prostate enzyme splits the liberated monoesters with consequent liberation of phosphate and inactivation of CoA. If this is true, then one can wonder why the split at the pyrophosphate bridge does not inactivate CoA for the test system. When this was studied, it was found that there is present an enzyme in the test system which resynthesizes the complete coenzyme from split products with ATP (table 6).

TABLE 5

Dependence of coenzyme A inactivation and phosphate liberation on order of addition of enzymes

FIRST ADDITION	SECOND ADDITION	CoA INACTIVATION	P LIBERATION
		%	%
Prostate enzyme	Potato enzyme	0	38
Potato enzyme	Prostate enzyme	100	100

TABLE 6

Resynthesis of potato enzyme-split coenzyme A by crude liver extract

	CoA ACTIVITY BY ASSAY	
	Purified system	Crude system
	Units/ml	*Units/ml*
CoA	2.4	43
CoA + crude enzyme	0.0	..
CoA + crude enzyme + ATP	39	43

Here we see that, if CoA is incubated with potato extract and its activity measured in the crude and purified assay systems, the crude assay responds as if to intact CoA, while the purified system shows little activity. If, however, the split product is first treated with the crude enzyme in the presence of ATP, then assayed in the purified system, the coenzyme is almost completely resynthesized. These observations, then, are consistent with the idea that the potato enzyme splits a pyrophosphate bridge in the coenzyme.

Experiments of a similar nature were performed with a highly purified sample of nucleotide pyrophosphatase pre-

pared from potato extracts and kindly furnished by Dr. Kornberg. This enzyme splits DPN, TPN, and FAD (flavine-adeninedinucleotide) at the pyrophosphate bridge (Kornberg and Pricer, '50). With CoA, essentially the same results were obtained as with the crude potato extracts already reported. One point should be made clear — that the potato enzyme attacks TPN, DPN, and FAD at pH 7.5, while the optimum for activity on CoA is at pH 4.5. This fact explains the failure of King and Strong ('51a) to obtain splitting of CoA by Kornberg's enzyme since those workers ran the reaction at pH 7.5.

TABLE 7

Liberation of muscle adenylic acid from coenzyme A by potato enzyme

	MOLAR RATIO		
	AMP-5′: PA	Free adenosine: PA	Total adenosine: PA
CoA	0.15	0.0	1.16
CoA + prostate enzyme	0.0	0.11	1.08
CoA + potato enzyme	0.6	0.33	1.12

ADENYLIC PORTION

If splitting by this potato enzyme is at the pyrophosphate bridge, then the resulting products should be some type of adenylic acid and a phosphorylated PA derivative. Accordingly, large enzyme preparations of the potato enzyme were made with the aim of identifying the degradation products. Unfortunately, the potato extract contains an acid phosphatase which is concentrated along with the pyrophosphatase. The presence of this enzyme makes the data somewhat difficult to interpret. However, by examining such an enzymically treated CoA, we were able to demonstrate the presence of muscle adenylic acid (table 7). The data in table 7 show that the CoA used for these experiments had a little free 5′-adenosinemonophosphate (AMP-5′) and a total adenosine content of 1.16 μM. After treatment with prostate phosphatase the free AMP-5′ is converted to adenosine by dephosphorylation, but most of the adenosine is still bound. After treatment with potato extract there is the appearance of 0.6 μM of AMP-5′,

while some of the liberated AMP-5′ has been converted to free adenosine by phosphatase action.

From these data it may be concluded that the pyrophosphate bridge emanates from position 5′ on the adenosine and is attached on the other end to pantothenic acid. Recently, somewhat better evidence for locating the pyrophosphate bridge has been obtained. On Kaplan's suggestion that snake venom contains a pyrophosphatase for DPN, attempts to repeat the potato enzyme experiments with snake venom have been made. Snake venom has, in addition to the pyrophosphatase, a specific phosphatase for AMP-5′. However, according to Kaplan, this enzyme does not attack adenosinediphosphate (ADP) as obtained from TPN. Accordingly, CoA was split with snake venom in conjunction with prostate phosphatase, and phosphate liberation was measured. The results are presented in table 8. Here we see that the CoA preparation contains practically no free phosphate and that, after acid digestion, approximately 3 phosphate groups per mole of PA are liberated. If the coenzyme is treated with snake venom alone, there is practically no liberation of phosphate although activity measurements indicate a complete splitting of the pyrophosphate bridge.

TABLE 8

Phosphate liberated from coenzyme A by various treatments

TREATMENT	μM P/μM CoA
None	0.02
Acid digestion	2.80
Snake venom	0.15
Prostate	1.02
Prostate// snake venom [a]	1.70
Snake venom// prostate [a]	2.60
Alkaline phosphatase	2.84

[a] See text.

When the coenzyme is treated with prostate phosphatase alone, one-third of the total phosphate is liberated, indicating the presence of a monoester. If the preparation is treated with prostate phosphatase first, then boiled, and subsequently

treated with snake venom, approximately two-thirds of the total phosphate is liberated, or 1 mole in addition to the free monoester. Again, activity measurements showed that the phosphorylated PA fragment was still intact, hence the extra phosphate did not arise from this portion. Now, since the snake venom has an AMP-5′ phosphatase, one could presume that the prostate enzyme removes a phosphate group from adenylic acid such that, after cleavage of the pyrophosphate bridge, AMP-5′ is liberated. This is subsequently attacked by the snake venom phosphatase to liberate the extra phosphate group. Therefore, when snake venom acts alone, it must be splitting off an ADP somewhat similar to that obtained from TPN by potato nucleotide pyrophosphatase.

If snake venom is allowed to act first and then prostate phosphatase, there is a complete liberation of phosphate, indicating that the action of the snake venom enzyme is to convert the diesters into monoesters which are subsequently attacked by the monoesterase in the prostate preparation. This is exactly what would happen if a pyrophosphate bridge were split. Likewise, alkaline phosphatase liberates all phosphate from CoA.

Confirmation of the fact that snake venom splits CoA into ADP and a phosphorylated PA derivative lies in an experiment in which the product was chromatographed, located under an ultraviolet lamp, and found to contain a ratio of phosphate to adenine of 1.85 : 1. These data, therefore, locate the free phosphate group of CoA on the adenosine moiety. Baddiley (personal communication) has confirmed this observation by periodate titration of CoA, from which he concludes that position 2 or 3 of adenosine is occupied. Kaplan has done some experiments to further locate this phosphate group, which he will probably explain in the discussion period.

THE NATURE OF THE PHOSPHORYLATED PANTOTHENIC ACID DERIVATIVE

If the structure of CoA is as we have proposed in figure 1, then the action of both the potato and the snake venom en-

zymes should result in the formation of a phosphorylated LBF, or phosphopantethine. A method for the investigation of this problem became available after Cheldelin and his co-workers observed that a bound form of PA, pantothenic acid conjugate, different from CoA, was vastly superior to the free vitamin in supporting growth of *Acetobacter suboxydans* (King et al., '48). Shortly thereafter we observed that CoA, as well as a variety of its enzymatic split products, supported better

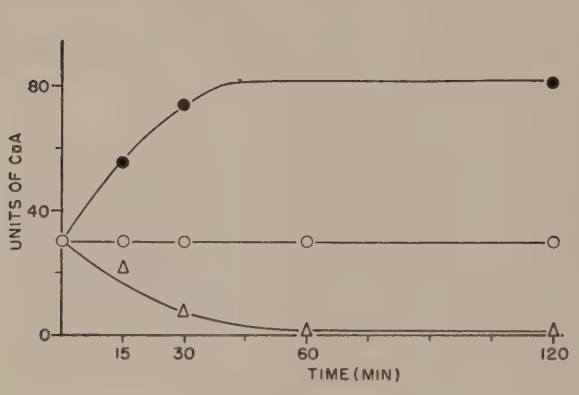

Figure 4

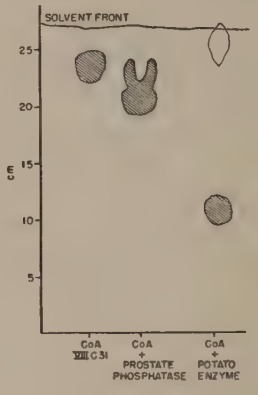

Figure 5

Fig. 4 Time curve of potato enzyme activity on CoA as measured by three assay methods.

● = Acetobacter assay ○ = Crude assay △ = Purified assay

Fig. 5 Chromatogram of enzymically treated CoA.
System: isoamyl alcohol
M/10 ammonium acetate (pH 8.1)
Shaded area = Ultraviolet Open area = Biological activity

growth than did the vitamin (Novelli et al., '49). In particular, the potato split product (or phospho-LBF) was even better than the intact coenzyme (Novelli et al., '50). This fact is illustrated in figure 4. Here we see that, as the potato enzyme activity progressed, as evident from the loss in activity in the purified assay system, the activity for *A. suboxydans* increased. The fact that the crude assay did not show a loss in activity is still another example of the resynthesis of this split product. The growth-promoting activity of

this split product is lost by its subsequent dephosphorylation with prostate phosphatase.

A clear picture (shown in fig. 5) results when the products of the reaction are chromatographed. Here we see that the growth activity for *A. suboxydans* has been completely separated from ultraviolet absorption. The eluted spot was also shown to be capable of undergoing resynthesis to the intact coenzyme.

In separate experiments it was shown that treating CoA with 1 *N* hydrochloric acid at 100°C. for 10–20 minutes resulted in cleavage of the pyrophosphate bridge. This suggested a means of preparation of phospho-LBF for isolation and characterization. Accordingly, after acid hydrolysis the product

TABLE 9

Analysis of barium salt isolated from coenzyme A by acid hydrolysis

SAMPLE	MOLAR RATIO			ACTIVITY	
	P: PA	Ribose: PA	Adenine: PA	Liver	Arsenolysis
				Units/mg	*Units/mg*
149–MEB–5	3.6	2.0	1.7	170	170
Ba–3	2.25	1.14	0.0	88	0
BaP1C	1.22	0.0	0.0	94	0

was isolated as the barium salt. The analysis of such a product is given in table 9. The data of this table show that the isolated barium salt was free from adenine and ribose and contained 1 mole of phosphate per mole of PA. Also the product was capable of undergoing resynthesis as evident from its activity with the liver assay. Finally, such a product was tested for its growth-promoting ability in *A. suboxydans.*

Table 10 compares the relative growth response of *A. suboxydans* to CoA and various split products. It can be seen that the isolated barium salt has the same activity as the potato split product, and that the activity is greatly diminished by dephosphorylation. These experiments indicate that, in addition to ADP, phospho-LBF is the other product of pyrophosphatase activity and may be the *Acetobacter* stimulatory factor. In this connection, recent experiments by Levin-

tow (unpublished observations) on the enzymatic synthesis of phospho-LBF are of interest. It had been observed earlier by King and Strong ('51b) as well as by Govier and Gibbons ('51) that synthetic pantethine could be synthesized to CoA by crude pigeon liver extracts. Levintow (unpublished) has purified this system and separated out an enzyme which performs the first step in this reaction, namely the phosphorylation of pantethine. The product of this enzymatic synthesis appears to have growth-promoting ability for *A. suboxydans* identical with that of the product of potato extract- or snake venom-splitting enzymes.

TABLE 10

Response of Acetobacter suboxydans to acid split product of coenzume A

TREATMENT	RELATIVE RESPONSE
CoA	100
CoA + potato enzyme	100
CoA + potato + prostate enzymes	33
CoA + alkaline phosphatase	31
Ba–5B	105
Ba–5B + prostate enzyme	45
Ba–5B + phosphatase	38
LBF	38
Pantethine	38

Baddiley and Thain ('51a) have studied by paper chromatography the products of mild acid or alkaline hydrolysis of CoA, and have presented evidence suggesting that the phosphate group is attached to the 4′ hydroxyl of pantothenate. These data, therefore, suggest that phospho-LBF is, in fact, 4′-phosphopantethine. There is, however, a series of observations which are not yet consistent with this formulation.

It would appear that, if phosphopantethine is the *Acetobacter* stimulatory factor, 4′-phosphopantothenate should have activity for this organism, especially since earlier observations (Novelli et al., '49) suggested that the β-mercaptoethyl-

amine fragment on CoA was not required for growth stimulation by this organism. Accordingly, Baddiley and Thain ('51b, c) synthesized the 4′ and 2′ phosphates as well as the cyclic phosphate which, when tested for activity with *A. suboxydans,* were found to be totally inactive. This finding was independently confirmed by King and Strong ('51b, c) who synthesized both phosphates as well as the diphosphate, and likewise found them inactive in supporting growth of *A. suboxydans.*

A possible explanation for these conflicting observations may be that the presence of the β-mercaptoethylamine moiety may be required to make phosphopantothenate permeable. A direct test of this suggestion will be possible as soon as 4′-phosphopantethine can be prepared synthetically. Until this question is resolved the location of the attachment of the pyrophosphate bridge must remain open.[1]

In summary, the available evidence suggests that CoA has the structure depicted in figure 1, with reservations concerning the exact position of the free phosphate group on the adenosine moiety and of the attachment of the pyrophosphate bridge to pantothenic acid.

DISCUSSION

KAPLAN: I should like to mention some work done in our laboratory by Schuster and Wang. Schuster has isolated an enzyme from barley which is a specific *b* nucleotidase. This enzyme will attack only *b* nucleotides. We tried a number of *b* purine and pyrimidine nucleotides (furnished through the generosity of Dr. Waldo Cohn) and found that all were attacked by the enzyme. On the other hand, the enzyme attacks none of the *a* and 5′ nucleotides.

Wang has tried the action of this enzyme on CoA with respect to the free phosphate and, interestingly enough, it

[1] Since this manuscript was submitted for publication, the presence of the pyrophosphate bridge as well as its attachment to the 4′ hydroxyl of pantothenic acid has been definitely established (Baddiley et al., '53; Novelli and Hoagland, '53).

did split off the phosphate group. The following is an experiment done with this 90% coenzyme A which Dr. Lipmann and Dr. Novelli sent us. Here it is shown that incubation of the coenzyme A with the *b* enzyme produces a stoichiometric amount of phosphate release — that is, 1 mole of phosphate per mole of CoA is liberated (as shown in the following tabulation).

	INORG. P (μM)	ARSENOLYSIS OF ACETYL-P UNITS	ACETYLATION OF SULFANILAMIDE UNITS
CoA (0.05 μM)	0	14.8	14.8
CoA (0.05 μM) + *b* nucleotidase	0.052	0	13.9

As Dr. Novelli has mentioned, the arsenolysis reaction is completely knocked out by removal of the free phosphate; whereas, the acetylation of sulfanilamide is not affected. These results are of the same type as those obtained with the prostatic treatment of CoA.

Dr. Wang has also found that TPN is not split by the *b* enzyme. You remember, Kornberg has found that the phosphate grouping in TPN is an *a* grouping. It looks, from this type of data, as if the free phosphate in coenzyme A differs from the free phosphate group in TPN. Wang has found, after snake venom pyrophosphatase action, that the diphosphoadenosine product coming from TPN moves, on paper, differently from the product formed from CoA.

He has also found that he can get stoichiometric release of 5′-adenylic acid by incubating diphosphoadenosine from CoA with the barley enzyme: he has measured the 5′-adenylate on paper, and by the specific muscle adenylic acid deaminase.

The thing that has not been done as yet is the identification of free *b* adenylic acid from the diphosphoadenosine fragment. By splitting off the 5′ group, one would hope to get the *b* adenylic acid, and this can be done only with a specific 5′ nucleotidase. As Dr. Novelli has pointed out, the snake venom

does not split diphosphate compounds; and this is also true of a specific 5′ enzyme from bull semen which Heppel has purified.

Our only hope seems to be on the potato enzyme which Kornberg found would split off the 5′ grouping from the diphosphoadenosine formed from TPN. We are now trying to get out the *b* adenylic acid from the diphosphoadenosine fragment of CoA.

Friedkin: Does the taka-diastase deaminase act on CoA?

Kaplan: This enzyme acts on CoA at a relatively slow rate. If the phosphate is removed with specific *b* phosphatase, a much faster rate is obtained. The difference between CoA and TPN with this deaminase is that the CoA is deaminated at a slow rate, whereas TPN is not deaminated at all.

Strehler: Has anyone hydrolyzed the peptide bond in the phosphorylated form of pantothenic acid to see whether the phosphate is actually attached to the pantoic acid residue, or whether it might be attached somewhere else on the molecule, for example, on the peptide nitrogen?

Novelli: In that connection, the Wisconsin group synthesized phosphopantoic acid as well as the phosphorylated pantothenic acids, and they also were inactive. It would be a little difficult to hydrolyze the phosphorylated compound and test it because pantoic acid itself is not very active in this organism.

Lipmann: Initially, we thought, quite understandably I believe, that the three phosphates in CoA were analogous to the phosphates of TPN. But Kaplan's results show now that the third phosphate in CoA is differently located. It is difficult to visualize, but I feel it worth while to ask what such a specificity of the linking of the third phosphate actually may mean in terms of activity.

More generally, I feel always tempted to emphasize the presence of these pyrophosphate bridges in so many coenzyme dinucleotides, while there is no indication of pyrophosphate links in nucleic acid.

COHN: When Volkin and I treated ribonucleic acid with snake venom, two different pyrimidine diphosphates were obtained. These diphosphates seemed to us to be *a*,5′ originally, but as we get them they are usually a mixture of *a*,5′ and *b*,5′, that is, 2′,5′ and 3′,5′. We think that is due to the acid-catalyzed conversion of some of the *a* form into *b*. We have not seen any pyrophosphates, however.

LAMPEN: Dr. Novelli mentioned, in discussing the nature of the sulfur compound in CoA, that this material had, initially, both the chemical reactivity of cystine and also microbiological activity. Was that due to contaminating cystine in those preparations, or does the β-mercaptoethylamine actually have activity in your microbiological assay?

LIPMANN: Gregory believes that cystine is a contaminant. There is convincing evidence that cysteine attaches itself to CoA through oxidative cross-linking during isolation. Such cross-linkings have likewise been observed with panthethine by Snell's group. More recently, I understand, Gunsalus' group is finding similar cross-linked contaminations with the lipoic acid. This type of contaminant is important to keep in mind with all compounds containing -SH or -SS groups. They are, of course, chemically linked to the compound and therefore represent "impurities" of a rather special type. They can be removed only by pushing the compound through a reduction step.

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THE ENZYMATIC SYNTHESIS OF ACYL-COENZYME A COMPOUNDS

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TWO FIGURES

Since the original discoveries that coenzyme A (CoA) is necessary for the acetylation of aromatic amines (Lipmann, '45) and of choline (Nachmansohn and Berman, '46) an abundance of evidence has accumulated indicating that acetyl-coenzyme A serves as an intermediate acetyl group carrier between various acetyl acceptor enzyme systems. The evidence in support of this generalized concept has been made the subject of comprehensive reviews by leading workers in the field (Lipmann, '50; Ochoa, '51; Barker, '51), and will not be considered here in detail.

The present discussion is devoted to a consideration of recent studies on the enzymatic acylation of coenzyme A by the phosphotransacetylase, acetaldehyde dehydrogenase, and CoA-transphorase systems found in extracts of *Clostridium kluyveri*. These enzyme systems serve to illustrate three different general methods for the biosynthesis of acyl-CoA compounds. In addition to these, a 4th method, involving the synthesis of acyl-CoA complexes by reaction with adenosine-triphosphate (ATP) and acetate (or another suitable carboxyl acid) is also known to occur (Novelli and Lipmann, '50; Ochoa, '51; Chantrenne, '51; Chou et al., '50); however, the mechanism of this conversion is still not fully understood (Barker, '51) and it will not be considered here.

THE PHOSPHOTRANSACETYLASE SYSTEM

Several years ago Lipmann and Tuttle ('45a) observed that cell-free extracts of *Eschericha coli* and *Clostridium butylicum* catalyze the rapid interchange of acetyl-bound and inorganic phosphate. It was proposed that this was due to reversibilty of the previously described reactions (Koepsell and Johnson, '42; Utter and Werkman, '44) in which pyruvate, in the presence of orthophosphate, was converted to acetyl phosphate (acetyl-P) and formate in extracts of *E. coli*, or to acetyl-P, carbon dioxide, and hydrogen in extracts of *C. butylicum*.

$$\text{Pyruvate} + \text{orthophosphate} \xrightleftharpoons{\textit{E. coli}} \text{acetyl-P} + \text{formate} \qquad (1)$$

$$\text{Pyruvate} + \text{orthophosphate} \xrightleftharpoons{\textit{C. butylicum}} \text{acetyl-P} + CO_2 + H_2 \qquad (2)$$

However, the lack of dependence of this interchange on reactants other than acetyl-P and orthophosphate (i.e., pyruvate, formate, carbon dioxide, and hydrogen) indicated that other factors might be involved.

Several years later in Barker's laboratory, while studying the synthesis of fatty acids in extracts of *Clostridium kluyveri*, we observed that the addition of inorganic arsenate to the reaction mixture completely inhibited the reduction of acetyl-P to butyrate. This inhibition was shown to be due to the complete and almost instantaneous decomposition of acetyl-P in the presence of arsenate (Stadtman and Barker, '50). The catalytic effect of arsenate on the hydrolysis of acetyl-P brought to mind a similar effect of arsenate on the hydrolysis of glucose-1-phosphate by the glucose-transferring enzyme obtained from *Pseudomonas saccharophila* (Doudoroff et al., '47). Accordingly, it was postulated that the arsenolysis of acetyl-P was due to an acetyl-transferring enzyme catalyzing reaction (3).

$$\text{Acetyl-P} + X \rightleftharpoons \text{acetyl-X} + \text{orthophosphate} \qquad (3)$$

$$\text{Acetyl-X} + \text{arsenate} \rightleftharpoons \text{acetyl-arsenate} + X \qquad (4)$$

$$\text{Acetyl-arsenate} + H_2O \xrightarrow{\text{spontaneous}} \text{acetate} + \text{arsenate} \qquad (5)$$

$$\text{Sum: Acetyl-P} + H_2O \rightarrow \text{acetate} + \text{orthophosphate} \qquad (6)$$

The arsenolysis of acetyl-P could then be explained by the substitution of arsenate for orthophosphate [reaction (4)] leading to the formation of acetyl-arsenate which it is assumed undergoes spontaneous hydrolysis [reaction (5)]. The net result of the three consecutive reactions is the hydrolysis of acetyl-P [reaction (6)]. This view was strengthened by the demonstration that cell-free extracts of *C. kluyveri* catalyzed the equilibration of P^{32}-labeled orthophosphate with the phosphoryl group of acetyl-P as is predicted by reaction (3).

The postulated acetyl acceptor, X, in these reactions could be either a substrate, coenzyme, or, as was originally proposed, the enzyme itself.

In view of the accumulated evidence showing that CoA was generally implicated in biological acetylation reactions, the possible role of this coenzyme in the arsenolysis of acetyl-P was investigated. It was found that the removal of CoA from extracts of *C. kluyveri* by treatment with ion-exchange resins or with charcoal resulted in the complete loss of arsenolysis activity, which was restored by the addition of purified CoA preparations (Stadtman et al., '51). This suggested that CoA was the postulated acetyl acceptor, X, referred to in reaction (3). Accordingly, the enzyme might be visualized as catalyzing the transfer of the acetyl group from acetyl-coenzyme A to orthophosphate [reaction (7)] and it was called a phosphotransacetylase (more commonly referred to as transacetylase).

$$\text{Acetyl-P} + \text{CoA} \leftrightharpoons \text{acetyl-CoA} + \text{orthophosphate} \qquad (7)$$

Direct proof for the existence of the hypothetical acetyl-CoA was obtained by Lynen and Reichert ('51) who succeeded in isolating this substance from yeast juice. They showed that the isolated compound can be used to acetylate sulfanilamide by crude pigeon liver extracts in the absence of ATP and acetate or acetyl-P. Indirect evidence that acetyl-CoA is formed in the transacetylase system was obtained by showing that the Lynen and Reichert acetyl-CoA will replace the transacetylase system as an acetyl donor in the synthesis of citrate (Stern et al., '51).

Earlier attempts by the author to obtain direct evidence for the formation of acetyl-CoA in the transacetylase system were unsuccessful. However, recent advances in our knowledge regarding the structure of CoA and acetyl-CoA have provided tools for the direct demonstration of acetyl-CoA in the acetyl-P-CoA reaction. First was the simultaneous discovery by DeVries et al. ('50) and by Snell et al. ('50) that CoA contains a free sulfhydryl group. This was soon followed by the studies of Lynen and Reichert ('51) showing that acetyl-CoA is a thioester of CoA and acetic acid. In common with other thioesters, the compound isolated by them from yeast juice is stable to heat under mildly acid conditions but is readily hydrolyzed in alkali even at room temperature. They further observed that acetyl-CoA does not give an immediate -SH test with slightly alkaline nitroprusside reagents but that it reacts readily with nitroprusside after hydrolysis with strong alkali. Finally, of considerable interest from the experimental point of view was the discovery by Novelli ('51) that S-acetyl compounds react quantitatively with hydroxylamine to give acethydroxamic acids under the conditions previously described for acyl phosphate determination (Lipmann and Tuttle, '45). These properties of acetyl-CoA and other thioesters are summarized in table 1. The nonenzymatic transesterification shown in the last line of table 2 was discovered in the course of the present study and has been shown to be a general reaction of acetyl thioesters (Stadtman, '52b).

With these properties in mind, experiments were made to determine if acetyl-CoA accumulates in the transacetylase system. The reaction was followed by taking advantage of the fact that acetyl-CoA is relatively stable, whereas acetyl-P is extremely unstable to heat (it is completely hydrolyzed by heating at 100°C., 5 minutes, pH 3–7). Since acetyl-P and acetyl-CoA both react with hydroxylamine to form acethydroxamic acids, the conversion of acetyl-P to acetyl-CoA is associated with a decrease in amount of heat-labile compound (acetyl-P) and a corresponding increase in the amount of

heat-stable compound (acetyl-CoA) as estimated by the hydroxamic acid method. Data presented in table 2 show that when acetyl-P is incubated with CoA in the presence of trans-

TABLE 1

Properties of acetyl-CoA and other thioesters

(1) Acetyl-S-CoA $\xrightarrow{100°C., pH 3–7}$ stable (little or no decomposition, 15 min.)

(2) Acetyl-S-CoA $\xrightarrow{\text{strong alkali}}$ acetate + CoA-SH

(3) Acetyl-S-CoA + $NH_2OH \xrightarrow{pH 7}$ $CH_3C(OH) = NOH$ + CoA-SH

(4) Acetyl-S-CoA $\xrightarrow[\text{mild alkali}^a]{\text{nitroprusside}}$ negative SH test

(5) Acetyl-S-CoA $\xrightarrow[\text{strong alkali or } NH_2OH]{\text{nitroprusside}}$ positive SH test

(6) Acetyl-S-CoA + RSH $\xrightarrow[\text{aqueous soln.}]{pH 7.5–9.0}$ acetyl-SR + CoA-SH

[a] Reagent 1 of Toennies and Kolb ('51).

TABLE 2

Influence of CoA on formation of heat-stable acetyl compound

CoA	HEAT-STABLE ACETYL		iP	μM HEAT-STABLE ACETYL
	60 min.	90 min.		μM CoA
$\mu M/ml$	$\mu M/ml$	$\mu M/ml$	$\mu M/ml$	
0.34	0.32	0.32		0.94
0.69	0.64	0.71		0.93
1.38	1.28	1.28		0.93
1.03		1.08	0.98	1.05

The reaction mixture contained 2.25 μM of acetyl-P, 50 μM of tris buffer (pH 8.0), 12 μM of hydrogen sulfide, 12 units of transacetylase, 20 μM of potassium chloride, and CoA (150 units per milligram) as indicated. Final volume, 0.5 ml, incubation, 90 minutes at 28°C.

acetylase, acetyl-P (i.e., heat-labile acetyl) disappears and equivalent amounts of heat-stable compound and inorganic phosphate are formed. Moreover, the amount of heat-stable compound formed is stoichiometric with respect to the amount of CoA added. Proof that the compound is acetyl-CoA was

obtained from another experiment in which C^{14}-labeled acetyl-P was incubated with CoA. After incubation, the reaction mixture was heated to decompose the excess acetyl-P and the heat-stable compound was isolated by paper chromatography. The developing solvent was a 1 : 1 mixture of ethanol and 0.1 *N* acetate buffer (pH 4.5). The newly formed compound was

TABLE 3

Quantitative analysis of acetyl CoA formed in transacetylase system

SAMPLE NO.	ACETYL-MERCAPTAN [a]	C^{14}-LABELED ACETYL [b]	CoA [c]	CITRATE PRECURSOR [d]	ACETYL DECOMPOSED ARSENOLYSIS [e]
	μM	*μM*	*μM*	*μM*	*μM*
1	0.27	0.24	0.28	0.27	
2	0.87		0.78	0.77	
3	0.42		0.30	0.38	
4	0.27			0.26	0.30
5				0.33	0.36

[a] Acetylmercaptan was measured by the hydroxamic acid procedure.

[b] C^{14}-labeled acetyl was estimated by C^{14} measurements.

[c] CoA was measured by the arsenolysis reaction.

[d] The test solutions contained 100 *μM* of tris buffer (pH 8.0), 2.5 *μM* of magnesium chloride, 30 *μM* of oxalacetate, 100 *μM* of potassium chloride, 35 units of crystalline condensing enzyme, and suitable aliquots of the acetyl CoA solutions; total volume, 1.0 ml. After 60 minutes incubation at 28°C., the reaction mixture was analyzed for citrate.

[e] Estimated by the decrease in hydroxamic acid-forming substance when incubated with arsenate and transacetylase.

Samples, 1, 2, and 3 represent samples of acetyl CoA isolated by paper chromatography (see the text). Samples 1, 2, and 4 were prepared from purified CoA preparations containing 150 units per milligram; samples 3 and 5 were derived from a CoA preparation containing only 13 units per milligram. Samples 4 and 5 were not purified by chromatography, but, instead, the reaction mixture was boiled 10 minutes at pH 5 to 6 to destroy excess acetyl-P and transacetylase. Sample 1 was prepared with C^{14}-labeled acetyl-P as described in the text.

identified on the chromatogram as a substance with an $R_f = 0.7 \pm 0.05$ (cf. CoA with $R_f = 0.55$). The compound appeared as a "quenching" spot when the paper was viewed under ultraviolet light; it reacted with hydroxylamine to give a hydroxamic acid; it gave a negative -SH test with the nitroprusside reagent 1 of Toennies and Kolb ('51), but after alkaline hydrolysis the test was positive. The compound was

eluted from the paper chromatogram and was found to contain equivalent amounts of CoA, C^{14}-labeled acetyl, and acetylmercaptan (table 3). The compound is enzymically active; it reacts quantitatively with oxalacetate to form citrate in the presence of Stern and Ochoa's condensing enzyme; it is arsenolyzed in the presence of transacetylase, and is used for the acetylation of sulfanilamide by aged extracts of pigeon liver. These results leave little doubt that the compound is acetyl-CoA and they confirm the findings of Lynen and Reichert ('51) that acetyl-CoA is an acetylmercaptan.

Spectrophotometric measurement of acetyl-CoA formation. More recently, we have followed the acetylation of CoA spectrophotometrically by measuring the increase in density at 240 mμ due to the formation of the thioester linkage.[1] Results of a typical experiment are given in figure 1. At zero time the reaction mixture contained acetyl-P and reduced CoA. After 5 minutes, transacetylase was added and there was an immediate increase in density due to the formation of acetyl CoA. The total density change was 0.095 Beckman scale divisions, which corresponds to almost complete acetylation of the reduced CoA added. After 15 minutes the addition of 20 μ*M* of potassium phosphate resulted in a decrease in density due to the conversion of acetyl-CoA to acetyl-P. After equilibrium was established (25 minutes), 100 μ*M* more of phosphate was added and the equilibrium was shifted to a new position due to the formation of more acetyl-P. The addition of arsenate at arrow 4 resulted in a decrease in density to the original starting point due to the complete arsenolysis of acetyl-CoA and acetyl-P. Finally, if at arrow 5 a large excess of acetyl-P is added, almost complete reacetylation of coenzyme A occurs as is indicated by an increase in density at 240 mμ to a point almost identical with the first density increase. The temporary complete acetylation of CoA in the presence of arsenate is possible because the rate of acetylation is much greater than the rate of arsenolysis. The data

[1] The author is indebted to Dr. E. Racker who called to his attention the fact that thioesters have an absorption band at 240 mμ.

of figure 1 serve to illustrate the freely reversible nature of the transacetylase reaction.

From experiments like that just described and from other experiments (Stadtman, '52b) that will not be described in detail, the equilibrium constant of reaction (7) has been determined to be 60–80 favoring acetyl-CoA formation. The free energy change is therefore about — 2000 to — 3000 calories,

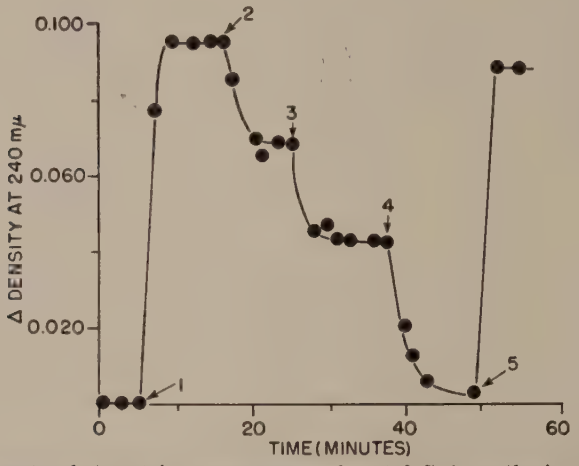

Fig. 1 Spectrophotometric measurement of acetyl CoA synthesis. Initially, the reaction mixture contained 1.2 μM of glutathione, 100 μM of imidazole-hydrochloric acid buffer (pH 6.8), 100 μM of potassium chloride, 0.22 μM of acetyl-P, and 0.086 μM of reduced CoA. Total volume 3 ml. Subsequent additions were made at times indicated by the arrows as follows: 1.5 units of transacetylase, arrow 1; 20 μM of potassium phosphate (pH 6.8), arrow 2; 100 μM of potassium phosphate, arrow 3; 10 μM of potassium arsenate, arrow 4; and 5 μM of lithium acetyl-P, arrow 5.

from which it can be estimated that the energy of the acetyl-S bond in acetyl-CoA is about 10,000–12,000 calories. This value is in agreement with that calculated from equilibrium data on the synthesis of citrate from oxalacetate and acetyl-CoA (Stern et al., '51; Stern and Ochoa, '52).

In the complete transacetylase system, the acetylation of various other mercaptans (viz., glutathione, thioglycolate, etc.) has also been observed (Stadtman, '51, '52b). This ace-

tylation is CoA dependent and is markedly influenced by the substrate and CoA concentration over wide ranges; the reaction is relatively independent on the concentrations of acetyl-P and transacetylase. It appears likely that the acetylation is due to a secondary nonenzymatic acetyl transfer from acetyl-CoA of acceptor mercaptan [reaction (8)].

$$\text{Acetyl-CoA} + \text{R-SH} \overset{\text{nonenzymatic}}{\rightleftharpoons} \text{acetyl-SR} + \text{CoA} \qquad (8)$$

This conclusion is supported by the finding that similar nonenzymatic acetyl transfers occur readily in dilute aqueous solution (pH 8), as for example the reaction between acetylthioglycolate and glutathione.

$$\text{Acetylthioglycolate} + \text{glutathione} \rightleftharpoons \text{acetylglutathione} + \text{thioglycolate} \qquad (9)$$

THE ACETALDEHYDE DEHYDROGENASE SYSTEM

Extracts of *C. kluyveri* catalyze the oxidation of acetaldehyde in the presence of orthophosphate to acetyl-P [reaction 10)] (Stadtman and Barker, '49).

$$CH_3CHO + HPO_4^{--} + \tfrac{1}{2}\,O_2 \rightarrow CH_3COOPO_3^{--} + H_2O \qquad (10)$$

This oxidation is analogous to the previously reported oxidation of pyruvate by extracts of *Lactobacillus delbrueckii* (Lipmann, '39).

$$CH_3COCOO^- + HPO_4^{--} \rightleftharpoons CH_3COOPO_3^{--} + CO_2 + 2H \qquad (11)$$

Following the suggestion of Negelein and Brömel ('39) who made a similar proposal to explain the oxidation of triose phosphate to 1,3-diphosphoglyceric acid, Lipmann postulated that pyruvate oxidation involved the addition of inorganic phosphate to the carbonyl group followed by oxidative decarboxylation of the semi-acetal intermediate [reaction (12)].

$$CH_3COCOO^- + HPO_4^{--} \rightarrow \left[\begin{array}{c} OPO_3^{--} \\ CH_3C\text{-}COO^- \\ OH \end{array}\right] \rightarrow CH_3COOPO_3^{--} + 2H + CO_2 \qquad (12)$$

An analogous sequence of reactions was also written for the oxidation of acetaldehyde (Stadtman and Barker, '49). This

explanation became uncertain, first as a result of the failure to obtain direct evidence for the hypothetical intermediate in triose phosphate and pyruvate oxidations (Henri and Fromageot, '25; Meyerhof and Junowicz-Kocholaty, '43), and subsequently, as a result of further studies on the oxidation of pyruvate to acetyl-P in various bacterial extracts. Strecker, Wood, and Krampitz ('50) observed that some enzyme preparations of *E. coli* possess the ability to catalyze the incorporation of formate into the carboxyl group of pyruvate under conditions where acetyl-P is not incorporated. The phosphoroclastic reaction [reaction (1)] might therefore be visualized as the sum of the two reactions:

$$\text{Pyruvate} + \text{X} \leftrightharpoons \text{acetyl-X} + \text{formate} \qquad (13)$$

$$\text{Acetyl-X} + \text{orthophosphate} \leftrightharpoons \text{acetyl-P} + \text{X} \qquad (14)$$

Rapid reversibility of reaction (13) could account for the formate exchange results, while irreversibility of reaction (14) or inactivation of the enymes involved would account for the failure of acetyl-P to be incorporated into pyruvate. This view received support by the experiments of Chantrenne and Lipmann ('50) showing that the formate exchange in extracts of *E. coli* was absolutely dependent upon the presence of CoA, and it was suggested that CoA was the postulated acetyl acceptor, X, in reaction (13). Subsequent experiments by Korkes et al. ('51) have shown that the oxidation of pyruvate by extracts of *Streptococcus faecalis* and *E. coli* is also CoA dependent, and they have obtained direct evidence that the formation of acetyl-P from pyruvate is a multistep process involving several different enzymes.

$$\text{Pyruvate} + \text{DPN}^+ + \text{CoA} \xrightarrow[\text{enzymes a, b}]{\text{cocarboxylase}} \text{acetyl-CoA} + \text{CO}_2 + \text{DPNH} + \text{H}^+ \qquad (15)$$

$$\text{Acetyl-CoA} + \text{HPO}_4^{--} \xrightleftharpoons{\text{transacetylase}} \text{acetyl-P} + \text{CoA} \qquad (16)$$

Reaction (15) is most probably the result of at least two separate reactions, since two protein fractions (a and b) were

needed in addition to the coenzymes; acetyl CoA was isolated as the end product (Korkes and Ochoa, '52, unpublished results). The enzyme-catalyzing reaction (16) was identified as transacetylase. These studies were extended to enzyme preparations derived from pig heart where an analogous series of reactions was found to occur (Korkes et al., '52).

In view of these experiments it seemed likely that the oxidation of acetaldehyde in *C. kluyveri* might proceed in a similar manner. The dismutation of acetaldehyde could be formulated as follows:

$$CH_3CHO + DPN^+ + CoA \xrightleftharpoons{\text{aldehyde dehyd.}} \text{acetyl-CoA} + DPNH + H^+ \quad (17)$$

$$\text{Acetyl-CoA} + HPO_4^{--} \xrightleftharpoons{\text{transacetylase}} \text{acetyl-P} + CoA \quad (18)$$

$$CH_3CHO + DPNH + H^+ \xrightleftharpoons{\text{alcohol dehyd.}} CH_3CH_2OH + DPN^+ \quad (19)$$

$$\text{Sum: } 2CH_3CHO + HPO_4^{--} \leftrightharpoons \text{acetyl-P} + CH_3CH_2OH \quad (20)$$

Recent studies in our laboratory by R. M. Burton ('51, '52) have provided experimental evidence for the above scheme. He has succeeded in obtaining a 20-fold purification of the aldehyde dehydrogenase from extracts of *C. kluyveri*. Data from his studies presented in table 4 show that the conversion of acetaldehyde to acetyl-P requires the presence of the three purified enzymes, aldehyde dehydrogenase, transacetylase, and alcohol dehydrogenase; in addition, CoA, diphosphopyridinenucleotide (DPN), inorganic phosphate, and glutathione (or some other sulfhydryl compound to convert CoA to the SH form) are obligatory requirements. These data are therefore consistent with the proposed mechanism [reactions (17), (18), (19)].

Other coupling systems such as glutamic acid dehydrogenase and lactic acid dehydrogenase, together with their respective oxidized substrates, will substitute for the alcohol dehydrogenase system. When the glutamic or lactic acid enzymes are used, either DPN or TPN will function as the intermediate electron carrier; the activity with DPN is about twice as great as the activity with TPN. It is not yet known

whether the lack of nucleotide specificity is due to a non-specific aldehyde dehydrogenase or is due to the presence of a mixture of DPN and TPN specific enzymes.

Proof for the sequence of reactions [(17), (18), and (19)] was obtained from spectrophotometric experiments with the purified enzymes. As shown in figure 2, incubation of acetaldehyde, CoA, and DPN with the purified acetaldehyde dehydrogenase results in the reduction of DPN as predicted by reaction (17). When equilibrium is reached, the addition of

TABLE 4

Oxidation of acetaldehyde to acetyl-P

COMPOUND OMITTED	ACETYL-P
	(μM/ml)
None (complete system)[a]	1.1
Acetaldehyde dehydrogenase	0.1
Transacetylase	0.2
Alcohol dehydrogenase	0.2
Coenzyme A	0.1
Glutathione	0.1
DPN	0.1
Potassium phosphate	0.1

[a] The complete system contained acetaldehyde, 100 μM; tris-hydrochloric acid buffer (pH 8.16), 0.2 M; glutathione, 5 μM; coenzyme A, 5 units; DPN, 0.1 μM; transacetylase, 9.5 units; yeast alcohol dehydrogenase, 260 units and acetylaldehyde dehydrogenase *(C. kluyveri)*, 1 unit. Volume, 1.0 ml., 30°C., 30 minutes.

inorganic phosphate and transacetylase at points 1 and 2 respectively, results in a further reduction of DPN due presumably to a shift in the equilibrium by the removal of acetyl-CoA through reaction (18). Finally, when a new equilibrium is established at point 3, the addition of a large excess of acetyl-P results in the complete reoxidation of DPNH, owing to a reversal of reactions (17) and (18). This sequence of events was further substantiated in separate experiments showing (a) that acetyl-CoA (isolated from the transacetylase reaction by paper chromatography) is reduced by DPNH in the presence of the aldehyde dehydrogenase, and (b), that

C^{14}-labeled acetyl-P is converted to C^{14}-labeled acetaldehyde in the presence of transacetylase, acetaldehyde dehydrogenase, CoA, and DPNH.

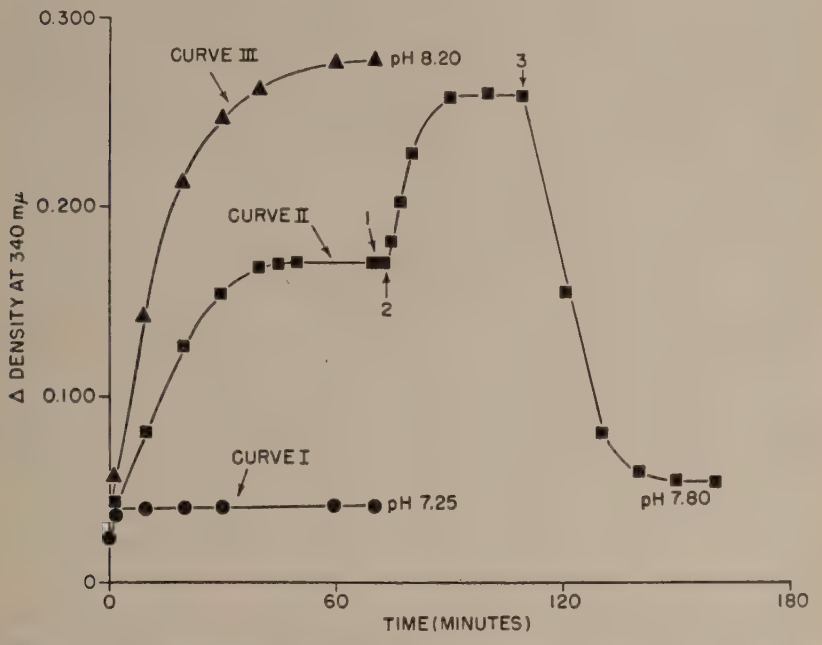

Fig. 2 The reversible oxidation of acetaldehyde to acetyl-P. All samples contained initially: acetaldehyde, 50 μM; tris-hydrochloric acid buffer at pH levels indicated, 200 μM; coenzyme A, 200 units; DPN, 0.8 μM; glutathione, 5 μM. At points 1, 2, and 3 (curve II) 2 μM of orthophosphate, 9.5 units of transacetylase, and 10 μM of acetyl-P, respectively, were added. Total volume 3 ml, room temperature (23–24°C.). The ordinate refers to optical density at 340 mμ as read in a Beckmann DU Spectrophotometer.

Concurrent with these investigations, studies by Pinchot and Racker ('51) have shown that the oxidation of acetaldehyde in extracts of *E. coli* is also CoA dependent.

THE CoA-TRANSPHORASE SYSTEM

It was recently discovered that extracts of *C. kluyveri* catalyze the conversion of acetyl-CoA to other acyl-CoA derivatives in the presence of higher fatty acids. This study was the outgrowth of a very early observation by Koepsell et al. ('44)

that extracts of *C. butylicum* catalyze the apparent phosphoryl group transfer from acetyl-P to butyrate forming butryl-P [reaction (23)]. Later, Lipmann and Tuttle ('44) demonstrated that extracts of *C. butylicum* catalyzed the reversible phosphoryl group transfers from acetyl-P and butyryl-P to ADP to form ATP. It was therefore postulated that the synthesis of butyryl-P from acetyl-P was mediated by ATP acting as an intermediate phosphoryl group carrier [reactions (21), (22), and (23)].

$$\text{Acetyl-P} + \text{ADP} \rightleftharpoons \text{ATP} + \text{acetate} \quad (21)$$

$$\text{ATP} + \text{butyrate} \rightleftharpoons \text{butyryl-P} + \text{ADP} \quad (22)$$

$$\text{Sum: Acetyl-P} + \text{butyrate} \rightleftharpoons \text{butyryl-P} + \text{ADP} \quad (23)$$

In the course of studies on the synthesis of fatty acids by extracts in *C. kluyveri* (Stadtman and Barker, '50), the preceding reactions were reinvestigated and the role of ATP as a phosphoryl group carrier was excluded by the finding that some enzyme preparations were unable to catalyze reactions (21) and (22) but they were able to catalyze reaction (23).

Later experiments carried out in Lipmann's laboratory (Stadtman, '50) showed that the removal of CoA from extracts of *C. kluyveri* resulted in the complete loss of the ability to form propionyl-P from acetyl-P and propionate and that this ability was restored by the addition of CoA.

Recent experiments in our laboratory have been made in an effort to determine the role of CoA in this reaction. At least two possible mechanisms were obvious:

Scheme A.

$$\text{Acetyl-P} + \text{CoA} \rightleftharpoons \text{CoA-P} + \text{acetate} \quad (24)$$

$$\text{CoA-P} + \text{propionate} \rightleftharpoons \text{propionyl-P} + \text{CoA} \quad (25)$$

$$\text{Sum: Acetyl-P} + \text{propionate} \rightleftharpoons \text{propionyl-P} + \text{acetate} \quad (26)$$

Scheme B.

$$\text{Acetyl-P} + \text{CoA} \xrightleftharpoons{\text{transacetylase}} \text{acetyl-CoA} + \text{orthophosphate} \quad (27)$$

$$\text{Acetyl-CoA} + \text{propionate} \xrightleftharpoons{\text{CoA transphorase}} \text{propionyl-CoA} + \text{acetate} \quad (28)$$

$$\text{Propionyl-CoA} + \text{orthophosphate} \xrightleftharpoons{\text{transacetylase}} \text{propionyl-P} + \text{CoA} \qquad (29)$$

$$\text{Sum: Acetyl-P} + \text{propionate} \rightleftharpoons \text{propionyl-P} + \text{acetate} \qquad (26)$$

In scheme A, CoA assumes the role of a phosphoryl group carrier between acetyl-P and propionyl-P.

In scheme B, on the other hand, CoA serves as an acyl group carrier only. Inorganic phosphate is formed in reaction (27) and is re-esterified in reaction (29). To differentiate between these possible mechanisms, the reaction was carried out with P^{32}-labeled acetyl-P and propionate in the presence of a large excess of unlabeled orthophosphate. The experiment was done with a *C. kluyveri* extract that had been heated for 30 minutes at 55°C. to cause preferential, but incomplete, inactivation of the phosphotransacetylase.

After incubation, the propionyl and acetyl phosphates were separated by paper chromatography and the orthophosphate by calcium precipitation. As shown in table 5 it was found that the specific activity of the propionyl-P was very nearly

TABLE 5

Formation of propionyl-P from propionate and orthophosphate in the presence of acetyl-P^{32}

REACTION MIXTURE			P^{32} AFTER 60 MINUTES		
Acetyl-P^{32}	Propionate	Ortho-phosphate	Acetyl-P	Propionyl-P	Ortho-phosphate
μM	*μM*	*μM*	*cpm/μM*	*cpm/μM*	*cpm/μM*
50	100	400	20,600	2960	1950
50	...	400	18,000	....	2240

Samples contained 10 *μM* of cysteine, 25 mg of enzyme and the indicated amounts of acetyl-P^{32}, propionate, and orthophosphate. Total volume, 1.0 ml, pH 6.9.

equal to that of the orthophosphate and was very much lower than that of the acetyl-P. These results prove that the reaction is not a direct phosphoryl-group transfer as postulated

in scheme A but that inorganic phosphate is formed as an intermediate. These data are therefore consistent with the postulated mechanism shown in scheme B.

Further proof for scheme B was obtained by the direct demonstration of reaction (28). Dialyzed extracts of *C. kluyveri* were incubated with acetyl-CoA and propionate or butyrate in the almost complete absence of orthophosphate (less than 0.5 μg per milliliter). After incubation the presence of propionyl-CoA and butyryl-CoA, respectively, was demonstrated by paper chromatography of the hydroxamic acid derivatives (table 6).

TABLE 6

CoA transfer from acetyl-CoA to propionate

REACTION MIXTURE				PRODUCTS [b]	
Acetyl-CoA	Propionate	Enzyme [a]	Pi	Acetyl-CoA	Propionyl-CoA
μM	*μM*	*mg*	*μM*	*μM*	*μM*
1.2	50	12	..	0.38	0.51
1.2	50	12	3	0.31	0.56
1.2	..	12	.	0.95	none
1.2	50	..	..	1.1	none

[a] Dialyzed enzyme in 0.83 ml of reaction mixture.

[b] Determined by paper chromatography of hydroxamic acid derivatives.

These results provide substantial support for a heretofore unrecognized type of CoA-linked reaction involving the transfer of CoA from acetyl-CoA to acceptor fatty acid. It was proposed that the enzyme responsible be called a CoA-transphorase (Barker, '51). The specificity of the enzyme is being investigated.

DISCUSSION

HORECKER: In your discussion of the acetyl-P to propionyl-P reaction, you said you used a preparation which had been heated, which destroys almost, if not all, of the transacetylase. Is transacetylase involved in the acyl phosphate interconversion system?

STADTMAN: I think it is; however we have no conclusive evidence on this point. The evidence that we do have is that in these heated preparations, the transacetylase activity is just sufficient to account for all of the transphosphorylation that occurs. However, the addition of transacetylase to such preparations does not accentuate the rate of the reaction. We have not been able to get any preparation that is completely devoid of transacetylase activity which still has the ability to catalyze the over-all acyl phosphate interconversion.

KAPLAN: You mentioned that the transfer of the acetyl group from acyl-CoA to various other compounds is not enzymatic. Is there any evidence of enzymatic transfer?

STADTMAN: Maybe I should not have emphasized the nonenzymatic character of the reaction. The evidence that it is nonenzymatic is based upon the observation that the reaction can proceed very well nonenzymically. It is possible, however, that there may be an enzymatic reaction as well.

KAPLAN: Is that freely reversible?

STADTMAN: Yes. You can get reactions between acetyl glutathione and CoA to give acetyl-CoA. We have observed similar reversible acetyl transfers between acetyl thioglycolate and glutathione and several other mercaptans. They occur nonenzymically in dilute aqueous solutions under exactly the same conditions of the enzyme studies (i.e., pH 8, 28°C.).

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THE MECHANISM OF THE ATP-CoA-ACETATE REACTION [1, 2]

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I wish to report here briefly on recent results on the so-called "ATP-acetate reaction," which we think reveal a rather unexpected mechanism. This conversion of the phosphate bond in ATP into the acetyl bond of acetyl ~ CoA — excluding acetyl phosphate as an intermediary — had so far been poorly understood. Quite a while ago, during Chou's work on this reaction with hydroxylamine as acetate acceptor (Chou and Lipmann, '52), we were puzzled by the observation that the hydroxamic acid formation was balanced by too little inorganic phosphate. Similar results were recently obtained using very active yeast fractions which had been prepared by Black in our laboratory. Again practically no inorganic phosphate appeared on incubation of the system ATP-CoA-acetate-hydroxylamine. This, however, was true only in the presence of fluoride, which had been used routinely to inhibit ATP breakdown. The present preparations contained relatively little ATP-ase and permitted us to carry out experiments without fluoride. On omission of the fluoride, surprisingly enough, the picture changed completely and hydroxamic acid formation was now accompanied by an abundant formation of inorganic phosphate. Indeed, in the absence of fluoride, about twice as much phosphate appeared as hydroxamic acid formed.

[1] A preliminary report by Lipmann et al. ('52) has appeared.

[2] The following abbreviations are used: ATP = adenosinepyrophosphate; AMP = adenosinemonophosphate; CoA = coenzyme A; Ac ~ CoA = acetyl ~ coenzyme A; Ac = acetate; ph = phosphate; and PP = pyrophosphate.

It was mainly this observation which gave away the explanation of the mechanism, namely, that not phosphate but pyrophosphate was liberated in the ATP-CoA-acetate reaction. It appeared that we had overlooked the relative abundance of pyrophosphatase in our yeast fractions. Yeast pyrophosphatase is strongly inhibited by fluoride (Kunitz, '52) and, for this reason, fluoride preserves the pyrophosphate. On the other hand, without the fluoride, initially formed pyrophosphate is split; this explains the appearance of two moles of inorganic phosphate for every mole of hydroxamic acid formed and ATP used, in contrast to a nonformation of orthophosphate with fluoride present.

The reaction therefore involves a pyrophosphate split of ATP, leaving AMP as the product of reaction:

$$\text{ATP} + \text{CoA} + \text{Ac} \rightleftharpoons \text{AMP} + \text{PP} + \text{Ac} \sim \text{CoA} \qquad (1)$$

In our experiments with hydroxylamine as acetyl acceptor, CoA acts catalytically, being recovered in the second and irreversible reaction:

$$\text{Ac} \sim \text{CoA} + \text{NH}_2\text{OH} \rightarrow \text{CoA} + \text{Ac-NHOH} \qquad (2)$$

This formulation is borne out by the results of the experiment shown in table 1. There, with fluoride present, ATP disappearance and hydroxylamine acetylation are balanced by a formation of AMP and pyrophosphate. Pyrophosphate was determined by the manganese precipitation of Kornberg ('50) and more rigorously identified by use of a crystalline preparation of pyrophosphatase (Kunitz, '52) kindly presented to us by Doctor Kunitz. AMP was determined spectrophotometrically (Kalckar, '47) using Schmidt's deaminase. ATP was assayed enzymically according to Kornberg's technique ('50) (to detect the disappearance of ATP, the enzymatic method had to be used). The easily hydrolyzable phosphate remained unchanged as was to be expected for a conversion of ATP to inorganic pyrophosphate.

Analagous experiments were carried out with pigeon liver extract where likewise, with the system ATP-CoA-acetate-hydroxylamine, equivalence of AMP and pyrophosphate for-

mation with disappearance of ATP and hydroxamic acid formation was observed.

In order to further clarify the mechanism, the possibility of reactions between (a) ATP and CoA and (b) between acetyl ~ CoA and pyrophosphate have been explored. Earlier experiments seemed to support the assumption of CoA-pyrophosphate as an intermediary (Lipmann et al., '52). Recent results, however, make us more cautious and leave still open the alternative of an enzyme-bound intermediary.

TABLE 1

Each vessel contained 29 μM ATP, 250 μM acetate, 860 μM NH_2OH (pH 6.5), 80 μM glutathione, 160 μM potassium fluoride, 640 μM tris-(hydroxymethyl)aminomethane buffer (pH 7.4), and 32 μM $MgCl_2$ in 3.2 ml total volume. Each vessel contained 0.32 ml of the yeast enzyme.

CoA UNITS	INCUBATION TIME	ATP	AMP	ACETHYDROXAMIC ACID	PYROPHOSPHATE
	min.	*μM*	*μM*	*μM*	*μM*
0	0	29.0	0.2	0	0
	150	22.9	1.2	2.7	0
290	0	28.7	2.8	0	0
	150	0.4	19.7	32.5	24.9
					23.1 [a]

[a] Value determined with pyrophosphatase.

The reversibility of the over-all effects could be shown most clearly in the following manner.

Acetyl phosphate was used with transacetylase as acetyl feeder to CoA. The thus-formed acetyl~CoA reacted with AMP and pyrophosphate to form ATP, while orthophosphate in place of pyrophosphate showed no reaction:

$$\text{Ac} \sim \text{ph} + \text{CoA} \xrightleftharpoons{\text{transacetylase}} \text{Ac} \sim \text{CoA} + \text{ph} \qquad (3)$$

$$\text{Ac} \sim \text{CoA} + \text{PP} + \text{ATP} \rightleftharpoons \text{ATP} + \text{CoA} + \text{Ac} \qquad (4)$$

Using yeast enzyme and transacetylase and applying methods similar to those described, the disappearance of 5 μM of acetyl phosphate was balanced by the appearance of 6.5 μM of ATP, while at the same time, 7.7 μM of AMP dis-

appeared. The experiment shows that, by way of acetyl ~ CoA and pyrophosphate, energy-rich acyl bonds may rather easily be converted to energy-rich phosphate bonds of ATP.

Recent studies here have shown that the pyrophosphate split of ATP appears to be a rather general reaction in intermediary metabolism. Maas and Novelli ('53) showed that the synthesis of pantothenic acid from pantoic acid, β-alanine and ATP with an enzyme in *Escherichia coli* occurs with liberation of AMP and pyrophosphate. Jencks (unpublished) in our laboratory, has furthermore observed an activation by ATP of longer chain fatty acids with liberation of pyrophosphate. It is significant that both these reactions appear *not* to require CoA. Therefore, the pyrophosphoryl split of ATP seems not to be specific for CoA-linked systems.

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THE CHEMISTRY AND FUNCTION OF THE PYRUVATE OXIDATION FACTOR (LIPOIC ACID)

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TWO FIGURES

This communication deals with two aspects of α-keto acid metabolism: first, the detection, isolation, identification, and synthesis of lipoic acid, a metabolite essential for oxidative decarboxylation of α-keto acids; and second, the enzymes and cofactors which function in keto acid dehydrogenation and decarboxylation and the mechanism of their reaction.

DISCOVERY AND DETERMINATION OF PYRUVATE OXIDATION FACTOR

The oxidative activities of *Streptococcus faecalis* cells, harvested from several growth media led to the recognition of a nutrient requirement for pyruvic acid oxidation which could not be replaced by any of the identified vitamins or coenzymes (O'Kane and Gunsalus, '48). For simplicity of reference and to denote the site of action, this nutrient was termed the "pyruvate oxidation factor" (POF) (O'Kane and Gunsalus, '48).

The pyruvate dehydrogenase and decarboxylase activities of *S. faecalis* were determined manometrically by either of the following reactions:

$$\text{Pyruvate} + 0.5O_2 \longrightarrow \text{acetate} + CO_2 \qquad (1)$$

$$2\ \text{Pyruvate} \longrightarrow \text{acetate} + CO_2 + \text{lactate} \qquad (2)$$

The first reaction was found to proceed via noncyanide-sensitive enzymes, with hydrogen peroxide as an intermediate which oxidized a second mole of pyruvate spontaneously to give the over-all reaction listed as reaction (1). The second

reaction corresponds to the stoichiometry of the Krebs dismutation.

Streptococcus faecalis, strain 10C1, grew almost equally well in a tryptone-yeast extract, phosphate-buffered medium with glucose as energy source, and in a synthetic medium in which hydrolyzed casein replaced the tryptone, and a mixture of vitamins and other growth factors replaced the yeast extract. The cells from these two media, however, behaved very differently toward pyruvic acid. The cells from tryptone-yeast extract medium metabolized pyruvate according to reactions (1) and (2) with a Q_{O_2} and a Q_{CO_2} of approximately 50, whereas cells from the synthetic medium possessed values of less than 5. Both types of cells oxidized glucose rapidly and at an equal rate ($Q_{O_2} = 60$). The addition of yeast extract to manometric cups containing cells from the synthetic medium initiated an immediate oxidation of pyruvate with a Q_{O_2} of about 50 — an observation interpreted as indicative of the presence of pyruvate "apodehydrogenase" in the cells grown in synthetic medium.

The formation of an apoenzyme by cells grown in the absence of the vitamin precursor of a "coenzyme" had previously been shown to occur for vitamin B_6. Data and interpretation from such experiments with both vitamin B_6-requiring amino acid systems and the pyruvate dehydrogenase system have been summarized by Gunsalus ('49).

The prompt activation of pyruvate apodehydrogenase by yeast extract indicated either the presence of POF coenzyme or its rapid formation by auxiliary enzymes in the cell suspensions (O'Kane and Gunsalus, '48). Acid-hydrolyzed yeast extract was also found to activate the oxidation of pyruvate by resting cells. This test system with proper adjuncts was adopted as an assay procedure for the purification of the "vitamin" form of the POF (Gunsalus, Dolin and Struglia, '52). More recently vacuum-dried cell preparations have been used for the same purpose (Gunsalus and Schnakenberg, unpublished data).

SYNONYMITY OF PYRUVATE OXIDATION FACTOR WITH OTHER UNIDENTIFIED GROWTH FACTORS

A comparison of stability, solubility, and adsorption properties of crude concentrates of POF with similar data for unidentified growth factors reported from other laboratories indicated a similarity to several such factors. An exchange of concentrates of several levels of purity followed by cross assays indicated the similarity of the pyruvate oxidation factor to the acetate-replacing factor (ARF) of Guirard et al. ('46) and to protogen, a growth factor for *Tetrahymena geleii* (Stokstad et al., '49; Snell and Broquist, '49). Subsequently, a similar relationship to a growth factor for *Butyribacterium rettgeri,* BR factor of Kline and Barker ('50), was shown (Gunsalus, Dolin and Struglia, '52; Kline et al., '52).

Following preliminary studies on the purification of the pyruvate oxidation (O'Kane and Gunsalus, '48; Gunsalus, Struglia and O'Kane, '52) and acetate-replacing factors (Guirard et al., '46; Reed . . . Johnston, '51), a crystalline substance possessing about 250,000 POF units (Gunsalus, Dolin and Struglia, '52) and 15×10^6 ARF units (Reed . . . Getzendaner, '51) per milligram has been obtained from hydrolyzed liver residue (Reed . . . Hornberger, '51) by the combined efforts of our group at Illinois, Reed and co-workers at the University of Texas, and the Research Laboratories of Eli Lilly and Company. This crystalline material, whose properties will be discussed has been characterized and named α-lipoic acid. The name, lipoic acid, now replaces the terms POF and ARF, previously used to denote biological activities, and refers to the first identifiable compound of this group of factors to be isolated. More recently the isolation of a crystalline derivative of one of the substances possessing protogen activity has been reported by Patterson et al. ('51).

MULTIPLE FORMS OF POF AND ARF ACTIVITY

Initial steps in the purification of the POF had led to the recognition of its stability to strong acid and alkali (O'Kane and Gunsalus, '48) and of the occurrence of five forms referred

to as a bound form, water soluble complex, weak acid, strong acid, and neutral form (Gunsalus, Struglia and O'Kane, '52). Reed and co-workers, who had taken up the purification of the acetate-replacing factor, had also recognized several forms of ARF by paper chromatography (Reed . . . Getzendaner, '51). Our cooperative efforts have identified the weak acid as the cyclic disulfide of an organic acid (α-lipoic acid), the strong acid as the sulfoxide of this cyclic disulfide (referred to as the β form), and the neutral form as the esters of these two acids. The interconversion of the cyclic disulfide and its sulfoxide have also been shown (Reed . . . Schnakenberg, '51). By methods similar to those used by Snell for the preparation of mixed disulfides of pantethine (Brown and Snell, '51), mixed disulfides between α-lipoic acid and several monothiols have been prepared (Reed, '52).

The "bound" and "water-soluble complex" forms of POF (Gunsalus, Struglia and O'Kane, '52) have been shown to yield lipoic acid.

ISOLATION, CHARACTERIZATION, AND SYNTHESIS OF LIPOIC ACID

As has been indicated, α-lipoic acid, a catalytic substance possessing pyruvate oxidation, and acetate-replacing factor activity, has been obtained from hydrolyzed liver residue as a crystalline material (Reed . . . Hornberger, '51). In view of the strongly acidic conditions used in preparation of α-lipoic acid, it is considered to correspond to a vitamin form of the catalytic agent required for pyruvate dehydrogenase activity. The isolation procedure developed for this form of the substance was based to a large extent on the acidic properties associated with the active principle. These steps included distribution between organic solvents (benzene, chloroform, ether) and aqueous phases of various pH's, and later on chromatography of the ethyl and methyl esters of lipoic acid on alumina and florisil, followed by mild alkaline hydrolysis to recover the

free lipoic acid. The crystalline α-lipoic acid was characterized by its X-ray diffraction pattern, a melting point of about 48°C., a pKa of 4.7 with a neutral equivalent of about 220. Quantitative analyses for carbon, hydrogen, and sulfur corresponded most closely to an empirical formula of $C_8H_{14}O_2S_2$, calculated equivalent weight 206 (Reed . . . Hornberger, '51; Reed . . . Schnakenberg, '51). Ultraviolet and infrared spectrophotometry indicated the absence of conjugated double bonds and of unsaturated linkages in the carbon chain. These data strongly suggested that α-lipoic acid is a cyclic disulfide of a C_8 fatty acid with positions α and β to the carboxyl unsubstituted. Further infrared spectra, reduction at the dropping mercury electrode and desulfurization with Raney nickel have shown: (a) the lack of a resolved methyl group at 3.4 μ, (b) a reversible polarigraphic half-wave potential and hydrogen-ion reduction potentials more nearly corresponding to cyclic six- or seven-membered than to five-membered rings, (c) normal octanoic acid chain, and (d) a rotation of + 97° in benzene (Reed . . . Gunsalus, '52).

These data are most compatible with the structure of α-lipoic acid corresponding to a cyclic disulfide of *n*-octanoic acid substituted on carbon-8, not on -2 or -3. Of the possible sites of attachment of the second sulfur, carbons-4, -5, or -6 must be considered as possibilities. If so, the molecule has one asymmetric carbon, the dextro rotatory isomer being the active substance.

Attempts to synthesize α-lipoic acid from 4-(α-tetrahydrofuryl)-butyric acid have yielded biologically active material (Hornberger et al., '52) as tested with the dried cell pyruvate dehydrogenase assay (Gunsalus and Schnakenberg, unpublished data). Attempts to prepare lipoic acid from tetrahydropyrane propionic and methyl tetrahydrofuryl propionic acids yield only bare traces of activity. These observations favor, but do not unequivocally demonstrate, that α-lipoic acid possesses a six-membered dithiane ring and is the cyclic disulfide

derived from 5,8-dithioloctanoic acid.[1] There is some tendency for carbonium ion migration and lactone formation after ring opening of the tetrahydrofuryl butyric acid.

ALPHA-KETO ACID DEHYDROGENASE SYSTEMS

Our knowledge of the α-keto acid dehydrogenase systems of animal tissue and of several types of bacteria have been reviewed recently by Ochoa ('51) and Vennesland ('51). Among the bacterial systems, I should like to mention briefly the studies of Lipmann ('39, '44; Chantrenne and Lipmann, '50) on the oxidative decarboxylation of pyruvic acid by *Lactobacillus delbrueckii;* the studies of Stumpf ('45) and, more recently, of Moyed and O'Kane ('51, '52), with *Proteus vulgaris;* the studies of Still ('41) on *Escherichia coli;* the fractionation of the pyruvate dehydrogenase system of *E. coli* by Korkes et al. ('50, '51) the description of an α-ketobutyrate dehydrogenase in *Streptococcus faecalis*, and the fractionation of the pyruvate and α-ketobutyrate dehydrogenases of this organism (Dolin and Gunsalus, '52). The recognition of the function of α-lipoic acid in the pyruvate dehydrogenase system of the latter organism by O'Kane and Gunsalus ('48) will be discussed subsequently, along with the documentation of the other cofactor requirements of the complete α-keto acid dehydrogenase systems.

Lipmann's ('39) early experiments on the pyruvate dehydrogenase of animal tissues were laid aside in favor of an analogous system in *L. delbrueckii* which exhibited greater stability. With the latter, he demonstrated for the first time acylphosphate formation with consequent inorganic phosphate requirement. A dependency upon thiaminpyrophosphate, magnesium, and flavineadeninedinucleotide were also reported. The *L. delbrueckii* system reacted with oxygen to form hydrogen peroxide or, anaerobically, conformed to the Krebs dismutation,

[1] Since this manuscript was submitted a report of the preparation of dithioloctanoic acid in which the second thiol group is on carbon-4, -5, or -6 has appeared; the isomer presumed to be the 6,8-dithioloctanate has been shown to be the biologically active compound (Bullock et al., '52).

reaction (2). Recent experiments with the *L. delbrueckii* system, based on Lipmann's demonstration of the acyl transfer function of coenzyme A, indicate that this system may differ from the pyruvate dehydrogenase found in several other bacteria and in animal tissues (Chantrenne and Lipmann, '50).

Stumpf obtained the pyruvate dehydrogenase of *Proteus vulgaris* in a cell-free state and demonstrated a dependency upon thiaminpyrophosphate and a divalent metal ion, of which manganese was the most active. In contrast to the *L. delbrueckii* system, as outlined by Lipmann, *Proteus vulgaris* yielded acetate and carbon dioxide as products of oxidation and did not show a phosphate dependency. Moyed and O'Kane ('51) have extended the studies of the pyruvate dehydrogenase of *Proteus vulgaris* and separated the system into two fractions — a clear protein fraction which appears to contain the decarboxylase, and an opalescent particulate fraction which appears to contain the electron acceptor function of the system (Moyed and O'Kane, '52). In agreement with Stumpf, these investigators found no inorganic phosphate requirement, and acetate, not acetyl phosphate, is the oxidized product. Coenzyme A does not appear to be essential for this system, but in the presence of added CoA, an acyl donor function can be shown by the acetylation of sulfanilamide or by the formation of citrate in the presence of added condensing enzyme (Ochoa et al., '51). A similar CoA-dependent acyl donor function was shown to occur in the *E. coli* and *S. faecalis* pyruvate dehydrogenase systems (Korkes et al., '50). Both *E. coli* and *S. faecalis* form acetyl phosphate during pyruvate dehydrogenation, according to reaction (3):

$$2\ \text{Pyruvate} + \text{inorganic phosphate} \rightarrow \text{lactate} + \text{acetyl phosphate} + CO_2 \quad (3)$$

The system of *E. coli* has been partially fractionated and shown to require four protein fractions (fractions A and B from *E. coli* precipitable respectively by 0.45 and 0.70 saturated ammonium sulfate; lactic dehydrogenase and transactylase). The latter two enzymes have been shown to serve the electron transport and acyl transport functions respectively. To date, precise functions have not been assigned fractions A

and B, but preliminary experiments by Korkes (personal communication), and in our laboratory (Gunsalus, Hager and Fortney, unpublished data) indicate that fraction A contains the carboxylase function of the pyruvate dehydrogenase system. Studies of this system are in progress, as well as attempts

TABLE 1

Pyruvate dismutation with purified E. coli enzyme fractions A and B

ADDITIONS	Δ MICROMOLES			
	Pyruvate	Lactate	Acetyl phosphate	Carbon dioxide
Complete	— 13.7	+ 5.7	+ 4.9	+ 6.0
No fraction A	— 0.2	+ 0.1	0	0
No fraction B		+ 1.2	+ 0.2	+ 0.1
No transacetylase	— 0.6	+ 0.3	+ 0.2	+ 0.1
No DPN				+ 0.5
TPN instead of DPN				+ 0.5
No CoA	— 1.6	+ 0.8	+ 0.3	+ 0.7

System, 1.5 ml containing: 100 μM of potassium phosphate buffer (pH 7.4), 2.5 μM of manganese chloride, 6.4 μM of L-cysteine, 0.15 μM of DPN, 0.2 μM of diphosphothiamine, 5 units of CoA, 40 μM of pyruvate, lactic dehydrogenase (2000 units), transacetylase (15 units), and enzyme fractions A (1.6 mg of protein) and B (1.9 mg of protein). Gas, nitrogen; incubation, 30 minutes at 25°C.

(Korkes, Del Campillo, Gunsalus and Ochoa, '51.)

TABLE 2

Dependence of pyruvate dismutation on lactic dehydrogenase

ADDITIONS	EXPERIMENT 1		EXPERIMENT 2	
	Δ CO_2	Acetyl phosphate	Δ CO_2	Acetyl phosphate
Complete	+ 2.2	+ 2.1	+ 2.1	+ 2.3
No transacetylase	+ 0.1	0		
No lactic dehydrogenase	+ 0.6	+ 0.1	+ 0.9	+ 0.6

(Korkes, Del Campillo, Gunsalus and Ochoa, '51.)

to separate further the enzymes and to characterize the single-step reactions of the pyruvate dehydrogenase system.

Measurement of the pyruvate dehydrogenase activity of the enzyme fractions from *E. coli* by dismutation [reaction (3)] also revealed multiple cofactor requirements, including: thiaminpyrophosphate, diphosphopyridinenucleotide (DPN), and

coenzyme A. The enzyme and coenzyme requirements are shown in tables 1 and 2. In an early experiment, an *E. coli* extract was dialyzed against *M*/40 pyrophosphate buffer at pH 8.6 for 24 hours at 5°C., to remove the thiaminpyrophosphate, and then against potassium chloride-cysteine to remove the pyrophosphate. Such extracts showed a tenfold stimulation in dismutation rate upon addition of cocarboxylase. Similar dependencies can be shown with condensing enzyme (Ochoa et al., '51) plus oxalacetate, to replace transacetylase and inorganic phosphate, as acetyl acceptor (Korkes et al., '51).

As these experiments show, the oxidative decarboxylation of pyruvate is, in fact, catalyzed by a system of enzymes with at

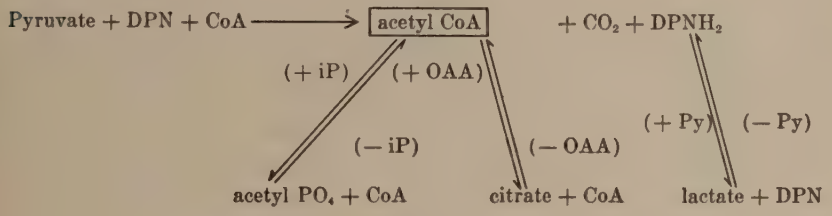


Figure 1

least three, and possibly four, cofactors mediating in the several single-step reactions. The data obtained with fractionated *E. coli* extracts have allowed a partial expression of the single-step reactions, the present visualization of which can be seen in figure 1.

The final steps are most clearly understood; i.e., an acetyl acceptor function mediated by transacetylase and inorganic phosphate, or by condensing enzyme and oxalacetate to yield acetyl phosphate or citrate with the regeneration of coenzyme A. Thus, catalytic amounts of CoA suffice to drive the reaction. Lactic dehydrogenase and residual pyruvate serve to regenerate DPN with the formation of lactate. The acyl donor function has been clarified by the recognition of acyl-CoA as a stable intermediate and its availability in substrate amounts. The DPN reductant of the system is unknown, as is the exact

nature of the decarboxylase reaction. These two steps constitute the focal problems of keto acid metabolism at this time. As a working guide, one may view the initial steps of decarboxylation and dehydrogenation as (1a) decarboxylation to an "aldehyde cocarboxylase complex," plus carbon dioxide, followed by (2a), dehydrogenation of this complex to an "acyl cocarboxylase complex," or (2b), a transfer of the aldehydo group to still another carrier, followed by oxidation to the acyl level.

Pyruvate $\longrightarrow$ aldehyde cocarboxylase complex + CO_2 (1a)

Aldehyde cocarboxylase complex + DPN $\rightleftharpoons$ acyl cocarboxylase complex + $DPNH_2$ (2a)

Aldehyde cocarboxylase complex + carrier $\rightleftharpoons$ cocarboxylase + aldehyde carrier complex

Aldehyde carrier complex + DPN $\rightleftharpoons$ acyl carrier complex + $DPNH_2$ (2b)

Although the evidence is scant, reactions (1a) plus (2b) seem at the moment to serve as the most useful model for study. If one is to consider the function of *E. coli* fractions A and B, keeping in mind that more than one enzyme may be present in each, fraction A would correspond to the decarboxylase. Evidence that fraction A contains the decarboxylase function is indicated by carbon dioxide exchange data (Korkes, personal communication), and by decarboxylation with fraction A in the presence of chemical electron acceptors (Gunsalus et al., unpublished data).

The α-keto acid dehydrogenase systems of *S. faecalis*, strain 10C1, have also been studied. These experiments were stimulated by the initial observation (O'Kane and Gunsalus, '48) that cells harvested from a synthetic medium require lipoic acid for pyruvate oxidation or dismutation. The specificity of lipoic acid for keto acid oxidation was indicated by an unimpaired rate of glucose oxidation of cells from lipoic acid-free media. Both cell suspensions and dried-cell preparations responded to free α- or β-lipoic acid (to their methyl or ethyl esters), and to more complex forms of the factor of which at least one is presumed to correspond to the coenzyme form

(Gunsalus and Schnakenberg, unpublished data; Gunsalus, Struglia and O'Kane, '52).

Data for the response of both cell suspensions and dried preparations to lipoic acid are shown in table 3. Both the oxidation [reaction (1)] and the dismutation [reaction (2)] are shown to require lipoic acid. Data are also included to

TABLE 3

Dependence of pyruvate and ketobutyrate dehydrogenases on pyruvate oxidation factor (lipoic acid)

ADDITIONS	O_2 UPTAKE		CO_2 EVOLUTION	
	Pyruvate	α-Keto-butyrate	Pyruvate	α-Keto-butyrate
	μl/hour	*μl/hour*	*μl/hour*	*μl/hour*
Resting cells:	6	20	21	6
(1) + Supplements[a]	12	15	22	10
(2) + 20 units α-lipoic acid	95	127	42[b]	50[b]
(1) + (2)	257	279	87	98
Dried cells:	30	24	29	12
(3) + Supplements	33	32	36	9
(4) + 20 units α-lipoic acid	150	145	81	72
(5) + 20 units β-lipoic acid	167	136	60	74
(3) + (5)	260	255	78	83

System, 2 ml containing: 3 mg dried apopyruvate dehydrogenase cells, 100 μM phosphate buffer (pH 6.5), 60 μM potassium keto acid.

Gas: air or nitrogen; 37°C.

[a] Per cup: 20 μg thiamin, 20 μg riboflavine, 200 μg adenosine, 5 mg potassium glutamate, 8 μM magnesium sulphate.

[b] + 2 μM manganese sulphate = 84 and 88.

show that α-keto butyric acid undergoes both the oxidation and dismutation reactions in preparations of *S. faecalis*, and that this reaction also requires lipoic acid as a cofactor.[2] Alpha-keto butyric acid was used as substrate after Juni (personal communication) found this keto acid not to function as a substrate for the acetoin system of *Aerobacter aerogenes*. As previously

[2] Lipoic acid has also been shown to be required for electron transport to ferricyanide with these cells (Gunsalus, Hager and Fortney, unpublished data).

shown (Dolin and Gunsalus, '51), extracts of *S. faecalis* harvested from either synthetic or tryptone-yeast extract medium, formed acetoin rapidly from pyruvate via the α-acetolactate acid pathway described by Juni ('52) for *A. aerogenes*. The acetoin-forming system *S. faecalis* has been shown to require cocarboxylase and divalent metal, but not to require lipoic acid (O'Kane, '50; Dolin and Gunsalus, '51). Thus, the function of lipoic acid must follow the decarboxylation of pyruvate. This reaction can be written as follows:

Pyruvate + cocarboxylase $\longrightarrow$ CO_2 + aldehyde cocarboxylase complex (4)

Aldehyde cocarboxylase complex + pyruvate $\longrightarrow$ α-acetolactic acid + cocarboxylase (5)

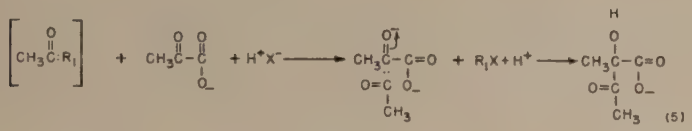
α-Acetolactic acid $\longrightarrow$ acetoin + CO_2 (6)

Sum: 2 pyruvate $\longrightarrow$ 2 CO_2 + acetoin

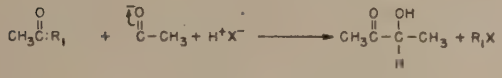
The "aldehyde cocarboxylase complex," reaction (4), is the intermediate step of the acetoin reaction, where a second molecule of pyruvate serves as the aldehydo (electron) acceptor to yield α-acetolactic acid. Thus, the departure of the routes of keto-acid metabolism would be dependent upon the nature of the aldehydo (and electron) acceptor at the aldehydo cocarboxylase stage.

The "aldehydo-cocarboxylase complex" pyruvate step, reaction (5), is presumed to have a higher dissociation constant than the initial carboxylase step, reaction (4), since the acetoin system of *S. faecalis* shows first-order kinetic behavior, and acetoin formation competes more favorably with the dehydrogenase reaction at high substrate concentration (Dolin and Gunsalus, '51). The Km for acetolactate decarboxylase, reaction (6), is 3×10^{-3}, whereas the Km for acetoin formation is 9×10^{-2}; therefore, the acetolactate carboxylase reaction cannot well account for the first-order kinetics or the high substrate requirement for acetoin formation. The condensation of the "aldehydo" with pyruvate to form α-acetolactate, reaction (5), and the analogous condensation of the "aldehydo" with

aldehyde to form acyloins directly, reaction (5a), may be visualized in greater detail as follows:



(5)



(5a)

where R_1 is assumed to be cocarboxylase. The "aldehydo" group of the "aldehyde cocarboxylase complex" can apparently serve also as the second aldehyde molecule in reaction (5a), although it is much less effective than free aldehyde. In this reaction, the aldehyde may be considered as an electron acceptor and, therefore, as the oxidant of the "aldehydo" group, just as ferricyanide serves as electron acceptor in (Schweet et al., '51) assay for pyruvate and ketoglutarate dehydrogenases of animal tissue. Similarly, lipoic acid coenzyme could serve as "aldehydo" and electron acceptor leading to acyl generation. Transfer of the acyl group to coenzyme A would lead to reduced lipoate, in turn reoxidized by DPN. The latter reaction would be analogous to the glutathione reductase of plant tissues as described by Mapson and Goddard ('51) and Conn and Vennesland ('51a, b). The E'_0 of lipoate in functional form is unknown; polarigraph data indicate a reversible equilibrium between α-lipoic acid (cyclic disulfide) and its dithiol (Reed et al., '53). With these observations as a basis, and α-keto butyrate, which does not undergo an acyloin condensation as substrate, the α-keto acid dehydrogenase of *S. faecalis* was followed by the dismutation assay (Korkes et al., '51). Further information on the mechanism of α-keto acid dehydrogenation and the mode of action of lipoic acid can be drawn from the effect of inhibitors upon the *S. faecalis* keto acid dehydrogenase system. The data in table 4 show a marked sensitivity of this enzyme to methylene blue heavy metals, and especially to arsenite. It is important also to note that the

inhibition by trivalent arsenicals is reversible by 1, 2 dithiols (BAL), but not by monothiols (glutathione or cysteine). The marked sensitivity to arsenite, 20 μM, and its reversal by BAL, compares directly with the data of Peters and co-workers ('46; Peters, '49) on the brain pyruvate oxidase. These workers observed a rather specific inhibition of brain pyruvate oxidase by Lewisite, and a reversal of this toxic action by BAL. In fact, they suggested the existence of a cyclic dithiol as a cofactor of the pyruvate oxidation system. Thus, the existence of lipoic acid as a catalyst of α-keto acid oxidation preceded its

TABLE 4

Inhibition of apopyruvate dehydrogenase and reversal with dithiols

ADDITIONS	CO_2 EVOLUTION (μl/hour)		
	No addition	+ BAL, 100 μg	+ GSH, 1 mg
None	26	33	25
α-Lipoic acid, 10 units	180	170	184
+ Arsenate, 10 mM	10	121	13
1 mM	130	126	
+ Arsenite, 0.1 mM	10	160	.
0.5 mM	12	170	20
0.01 mM	93	174	..
+ Methylene blue, 1 mM	0	101	0

Complete system, M/20 phosphate buffer (pH 6.0), 60 μM potassium α-ketobutyrate, 5 mg apopyruvate dehydrogenase dried cells. Gas: nitrogen, 37°C.

isolation and the demonstration of its cyclic disulfide nature by nearly 10 years. The arsenite inhibition and reversal by 1, 2 dithiols also furnishes indirect evidence for the mediation of the dithiol form of the lipoic coenzyme during its biological function.

Whole-cell preparations, particularly the dried cells from synthetic medium, have thus been extremely useful in establishing the function of lipoic acid in keto acid metabolism and in suggesting the existence of a dithiol form of this catalyst. Further clarification of the reaction sequence of keto acid dehydrogenation, the identification of the form of lipoic acid

which corresponds to the coenzyme,[3] and the measurement of equilibria or potentials, and thus the energy relationships, are dependent upon the separation and resolution of the keto acid dehydrogenase enzymes. With this objective in mind, cell-free extracts of *S. faecalis* were prepared, separated into several enzyme components, and the cofactor requirements of this keto acid dehydrogenase compared to those of *E. coli* and *L. delbrueckii.*

It has been possible to separate the *S. faecalis* system into two enzyme fractions, each inactive by itself, but highly active in the presence of the other fraction plus lactic dehydrogenase and suitable cofactors. The fractionation scheme used is shown in figure 2, data for thiamin dependence in table 5, and other cofactor and enzyme requirements in table 6. As will be noted, this system is in all respects similar to the *E. coli* pyruvate dehydrogenase dismutation system. Transacetylase has not been completely separated from the lower enzyme, fraction I, but is presumed to function since acyl phosphates are formed and coenzyme A is required in catalytic amounts. The transacetylase present in *S. faecalis* will catalyze the arsenolysis of propionyl, as well as acetyl phosphate. The rate of the former reaction is approximately one-half the acetyl phosphate arsenolysis rate. The data in table 7 show a stoichiometric formation of propionate, carbon dioxide, and α-hydroxy butyric acid. In later experiments with fractionated extracts, accumulated propionyl phosphate was identified by its paper chromatogram. The α-hydroxy butyrate was identified by a modification of the method of Barker and Summerson ('41). Fractions I and II, obtained by fractionating α-keto butyrate dehydrogenase activity, still contain an acetoin-forming system with pyruvate as substrate. In addition, the dismutation of pyruvate, as measured by acyl phosphate and lactate accumulations, is equal to the keto butyrate dehydrogenase activity. The similarity of the behavior of these two substrates, and the

[3] Two communications indicate that the coenzyme form of lipoic acid is an amide with cocarboxylase for which the term lipothiamidepyrophosphate has been suggested (Reed and DeBusk, '52a, b).

results of preliminary experiments with other α-carbonyl compounds, including other α-keto acids, indicate a general requirement of lipoic acid for the cleavage of carbonyl compounds and their dehydrogenation to acyl donors.

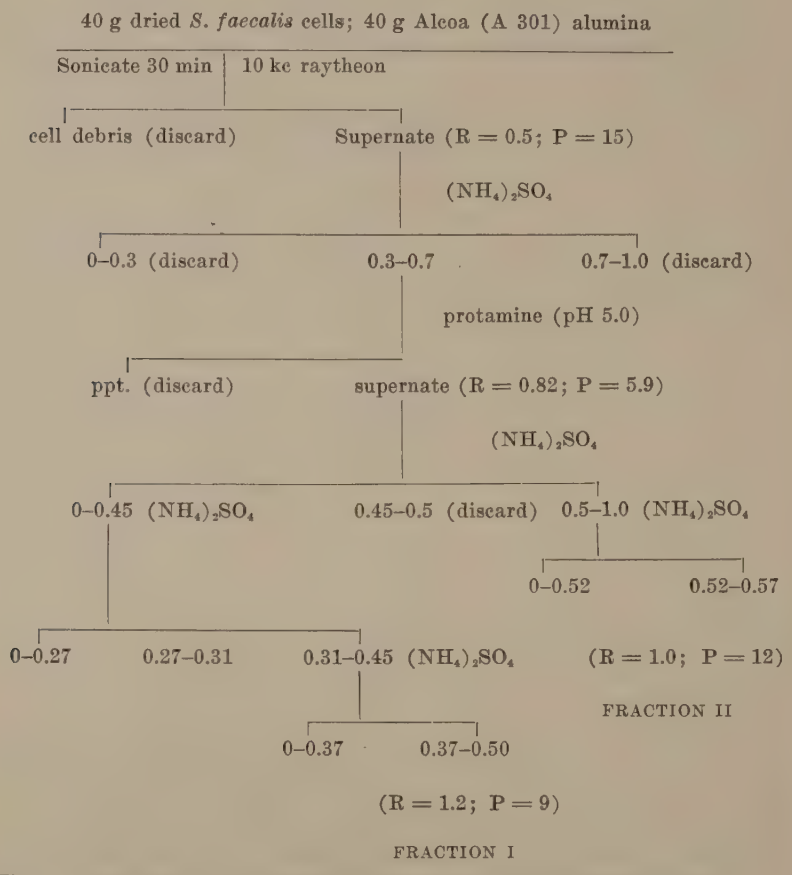


Fig. 2 Fractionation of pyruvate (ketobutyrate) dehydrogenase system.

In summary, the pyruvate oxidation factor has been isolated and characterized as a cyclic disulfide of octanoic acid. Its function in several α-carbonyl cleavage and dehydrogenation systems has been demonstrated. Evidence has been obtained for a dithiol stage in lipoic acid function.

TABLE 5

Requirements for α-ketobutyrate dismutation (Streptococcus faecalis 10C1, dialyzed crude extracts)

ADDITIONS	CO_2 EVOLVED	RESOLUTION
	μl/hour	%
1. Sonic extract, dialyzed vs. pyrophosphate		
Complete:	60	..
—cocarboxylase	18	70
2. Ammonium sulfate ppt. 0.3–0.7 sat., dialyzed vs. potassium chloride-cysteine		
Complete:	168	..
— phosphate	64	62

TABLE 6

Components of the pyruvate and α-ketobutyrate dismutation system Streptococcus faecalis (10C1) enzyme fractions

ADDITIONS	SUBSTRATE: PYRUVATE	SUBSTRATE: α-KETOBUTYRATE
	Lactate formed	CO_2 evolved
	μM	*μM*
Complete:	5.2	5.6
— cocarboxylase	2.4	2.8
— manganese	2.4	3.0
— DPN	0.9	2.0
— lactic dehydrogenase	0.75	5.0
— CoA	0.3	1.1
— cysteine	0.0	0.9
— enzyme I	0.0	0.20
— enzyme II	0.15	0.30

System, 2 ml containing: 200 *μM* potassium phosphate buffer (pH 6.0), 0.15 *μM* DPN, 0.2 *μM* cocarboxylase, 2 *μM* manganese sulphate, 10 units CoA (170 units/mg), 6.4 *μM* cysteine, 2000 units lactic dehydrogenase, 0.3 ml enzyme fraction I (2.3 mg protein), 0.4 ml enzyme fraction II (4.8 mg protein). Gas: nitrogen, incubation, 60 minutes at 35°C.

TABLE 7

Dismutation of α-ketobutyrate Streptococcus faecalis (10C1) sonic extract

Δ MICROMOLES			
α-Ketobutyrate	Carbon dioxide	Propionic acid	α-Hydroxybutyric acid
−175	+84	+81	+80

The pyruvate dehydrogenase systems of *E. coli* and *S. faecalis* have been fractionated, the individual steps indicated, and the function of several cofactors clarified. The role of lipoic acid in keto acid dehydrogenation has been localized to the reactions between cocarboxylase and coenzyme A.

DISCUSSION

Novelli: It was not clear to me from your slides whether the addition of lipoic acid to resting cells gave an immediate stimulation in pyruvate oxidation or if there was a lag period.

Gunsalus: This question has come up before. If α-lipoic acid is added to a system in which all the other ingredients have been equilibrated in the cup, an increase in oxidation rate will occur within 5 minutes and reach a maximum in 15 to 20 minutes. Vacuum-dried cells give the same response also with the short lag. I believe these observations rule out adaptive enzyme formation.

Novelli: Can the lag be eliminated by preincubation with lipoic acid?

Gunsalus: No.

Novelli: You said there was lipoic acid activity in your relatively purified enzyme system. Is there free lipoic acid present or is it as a coenzyme?

Gunsalus: It is not free lipoic acid, but a form which can be removed from the enzyme by hot water extraction. The lipoic acid activity recovered is not α- or β-lipoic acid nor their esters, but a more polar form, soluble in water but not organic solvents.

Novelli: Does the form of lipoic acid activity removed from the enzyme by hot water extraction contain phosphate?

Gunsalus: We do not know.

Chairman Lipmann: I do not quite understand how you supplied the lipoic acid.

Gunsalus: If I understand Doctor Novelli's question, he wants to know if a lipoic acid coenzyme exists, if it is formed from free lipoic acid by the preparation which oxidizes pyruvate, and if there is any evidence of enzyme formation in the

cell suspensions or preparations. We believe there is a coenzyme form of lipoic acid, and we do not believe lipoic acid functions in pyruvate dehydrogenase formation. As an example, a dried-cell preparation, grown free of lipoic acid, will oxidize pyruvate if lipoic acid is added. If the lipoic acid is incubated with the cells in the absence of pyruvate, virtually all of it can be recovered as free lipoic acid.

NOVELLI: We have essentially the same phenomenon with pantothenic acid in acetate respiration. There was a very long lag, initially. If we preincubated cells with pantothenic acid and glucose, then washed out the glucose, the lag was completely eliminated.

GUNSALUS: We are probably in a little better position. You had to add an energy source because acetate would not release energy. In our studies the pyruvate yields $\sim$ P as the reaction proceeds.

LIPMANN: It was not quite clear from one of your slides if you needed CoA in addition to the lipoic acid.

GUNSALUS: Present evidence would indicate that both are required in the *Streptococcus faecalis* system. There may be, however, other pathways, one of which may be very slow. I believe you were first to suggest that some of the thiol esters might bypass CoA and operate at slower rates. There is reason to believe this is the case. CoA is required, however, for the *S. faecalis* system. The coli preparations we have fractionated are partially CoA dependent in the sonic extracts — after the first ammonium sulfate fractionation the preparations are virtually free of CoA. The faecalis preparations do not show a CoA dependence when first extracted, however the ammonium sulfate-fractionated enzymes are CoA dependent.

LIPMANN: I understand that Green has some preparations where he gets an oxidation with ferricyanide and which seem not to need CoA or DPN.

GUNSALUS: We have studied this reaction. I will leave the ferricyanide data for Doctor Dolin to discuss if he wishes.

DOLIN: The only thing I want to say about the CoA is that, as Doctor Gunsalus mentioned, the original extracts show only

slight dependence upon added CoA, yet their CoA content is very low — about one-tenth of a unit per milliliter. After fractionation, the dismutation is stimulated about fivefold by the addition of CoA.

GUNSALUS: In the dismutation of pyruvate by these extracts, 2 pyruvate → acetate + lactate + CO_2 acyl phosphate formation is lower than the carbon dioxide formation. That is, there seems to be acetate formation which is not acyl. For this reason we have wondered if the so-called hydrolytic enzyme might act on an acyl substrate previous to CoA. Such an occurrence would explain Green's observation that acetate formation does not require CoA, and our observation of less acyl phosphate formed than one would expect from the carbon dioxide formation, and a negligible CoA requirement.

LIPMANN: I would like to make one more comment. We have frequently talked about the possibilities that *L. delbrueckii* might contain lipoic acid. This system seems not to require CoA, but when I listened to what he said, it occurred to me that it probably does not need lipoic acid because it is not arsenite sensitive.

GUNSALUS: I understand that O'Kane and Moyed also do not find an arsenite sensitivity for the *Proteus vulgaris* pyruvate dehydrogenase. Neither does their system appear to require CoA, and presumably it has no lipoic acid.

LICHSTEIN: Doctor Gunsalus made an important point, namely, that a factor is required for a specific function but not for growth.

As I understand it, this *Streptococcus* grows perfectly well without lipoic acid, and yet it does not have pyruvate oxidation activity. Recently, in studies with the formic dehydrogenase system, we have the same phenomenon occurring. We can grow cultures of wild strains of coli, or mutant strains of coli and aerogenes. They grow luxuriantly, and yet do not show the enzyme activity.

Perhaps it is worthwhile to re-emphasize the point that Doctor Gunsalus made briefly when he talked about whether or not α-lipoic acid is a true vitamin. I think perhaps we are over-

looking the fact that vitamins or growth factors may not be discovered if we use only growth studies. There is in the *L. delbrueckii* a factor replacing α-lipoic acid for the growth of the organism. For the streptococci, perhaps lipoic acid is not necessary at all for growth, and certainly the factors we are dealing with show no evidence of being required for growth. Yet cells can be obtained which are deficient in certain enzyme systems. I am merely making the comment because it seems to me that in the area of nutrition we should perhaps be using more of the enzyme assay, rather than just growth assay.

GUNSALUS: I have looked at this phenomenon in the sense that a cofactor is required by an organism when the cell must form some compound, or compounds. As an analogy, in the vitamin B_6 case, Snell was able to replace vitamin B_6 in the growth medium for *S. faecalis* with D-alanine. From this he deduced that D-alanine was a precursor of vitamin B_6. It turned out to be quite the converse: D-alanine is the only essential metabolite not furnished in the growth medium, for whose formation vitamin B_6 is required.

Cells grown in the presence of D-alanine did not seem to store vitamin B_6, but those grown in the presence of vitamin B_6 did contain D-alanine. In the present instance the pyruvate oxidation factor (lipoic acid) appears to function in acetate or acetyl formation — i.e., pyruvate to acetate. We add to our synthetic medium enzyme-hydrolyzed casein which acidified with acetic acid. Acetate is an essential metabolite, which can either be furnished or replaced by the catalysts for its formation.

Snell had first visualized the acetate-replacing factor to be some fatty acid for which acetate was a precursor. In fact, he had prepared a fraction from yeast which was 440 times as active as acetate in stimulating the growth of his test organism. In this case, we had considered the factor to be of catalytic nature — for pyruvate oxidation. The catalytic nature would explain the trace requirements. It turned out that we had to concentrate lipoic acid around 300,000-fold over the amount present in liver residue to obtain the crystalline material.

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THE METABOLISM OF PENTOSE PHOSPHATE

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SIXTEEN FIGURES

The material to be discussed in this paper will fall into two sections. The first will contain a brief review of work carried out in collaboration with Smyrniotis and Seegmiller on the oxidation of 6-phosphogluconate and the formation of the pentoses, ribulose-5-phosphate and ribose-5-phosphate (Horecker et al., '51; Seegmiller and Horecker, '52). This review will provide a framework for a discussion of the reversibility of this oxidation as well as the oxidation of glucose-6-phosphate. Together, these provide a mechanism for the fixation of carbon dioxide in hexoses. The second part will be a discussion of some current work with Smyrniotis on the further metabolism of the pentose phosphates. The results of these studies suggest the existence of a cyclic mechanism, in which glucose-6-phosphate is regenerated, which may be of considerable importance in carbohydrate metabolism.

Several years ago, we became interested in the metabolism of 6-phosphogluconate because it appeared to provide an interesting pathway for carbohydrate oxidation. The work of Lipmann ('33, '34), Lundsgaard ('30), and Shorr and his coworkers (Barker, Shorr, and Malam, '39) had suggested the existence of such a pathway, which might under certain conditions replace the glycolytic mechanism. Engelhardt and Barkash ('38) were of the opinion that this was an oxidative process which diverged from the Embden-Meyerhof pathway at the stage of glucose-6-phosphate. This is shown in figure 1. Glucose-6-phosphate, instead of undergoing phosphorylation and conversion to pyruvic or lactic acid, would be

directly oxidized. A few years earlier Warburg and Christian ('35) had shown that the product of glucose-6-phosphate oxidation with enzymes from yeast as well as from animal tissues was 6-phosphogluconate. In yeast extracts they found

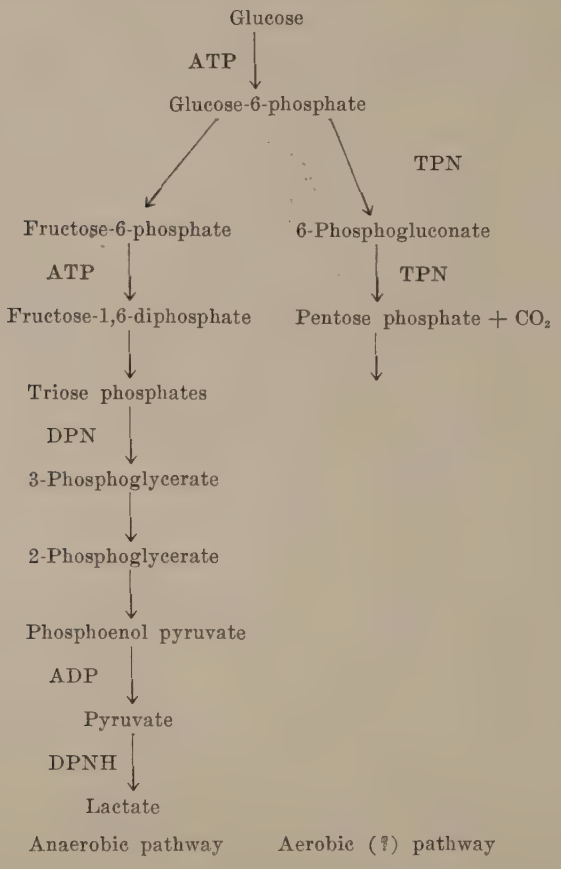


Fig. 1 Glucose-6-phosphate metabolism.

this product to be further oxidized with the evolution of carbon dioxide. The other products of the reaction were not identified. Dickens ('38a) studied the oxidation of phosphogluconate with enzymes from yeast with similar results. Among the products, he observed a substance giving the or-

cinol test for pentose. On the basis of the observation that these yeast preparations were able to oxidize ribose-5-phosphate rapidly, Dickens ('38b) concluded that this substance was an intermediate in phosphogluconate oxidation. Confirmation of this view was provided by Scott and Cohen ('51), who obtained evidence by chromatographic and microbiological methods that a ribose ester was formed in the oxidation of phosphogluconate with Dickens' yeast enzymes.

In order to obtain more definitive information on the nature of the intermediates in 6-phosphogluconate oxidation, we undertook the purification of the enzymes involved. It had already been established by Warburg and Christian ('37) that the coenzyme for phosphogluconic dehydrogenase was triphosphopyridinenucleotide (TPN). The enzyme was purified from yeast on the basis of the reduction of this coenzyme (Horecker

$$\text{6-Phosphogluconate} + \text{TPN}^+ \rightarrow \text{Pentose-5-phosphate} + CO_2 + \text{TPNH} + H^+$$
$$\text{Pyruvate} + \text{TPNH} + H^+ \rightarrow \text{Lactate} + \text{TPN}^+$$

Fig. 2 The TPN-coupled oxidation of 6-phosphogluconate by pyruvate.

and Smyrniotis, '51). The purified enzyme was useful not only for the preparation of the reaction products but also for the determination of 6-phosphogluconate, since 1 mole of TPN was reduced per mole of phosphogluconate added and this reduction could be measured spectrophotometrically.

To accumulate the reaction product without adding stoichiometric quantities of TPN, it was necessary to provide a mechanism for regenerating TPN from the reduced form (fig. 2). This was done by adding an excess of pyruvate and lactic dehydrogenase. Thus, the quantitative oxidation of phosphogluconate was effected with catalytic amounts of coenzyme. For each mole of phosphogluconate utilized, 1 mole of pyruvate was consumed and 1 mole each of carbon dioxide, pentose phosphate, and lactate were formed.

Attempts to establish the identity of the pentose phosphate reaction product soon led to the conclusion that more than one component was present. In paper chromatography two

pentose spots were observed and with periodate oxidation small but significant amounts of formaldehyde were produced which could not have arisen from ribose-5-phosphate or arabinose-5-phosphate, since these compounds contain no free primary hydroxyl groups. By means of ion-exchange chromatography the two pentose esters could be separated, as is shown in figure 3. The component with positive rotation corresponded in position and optical activity to ribose-

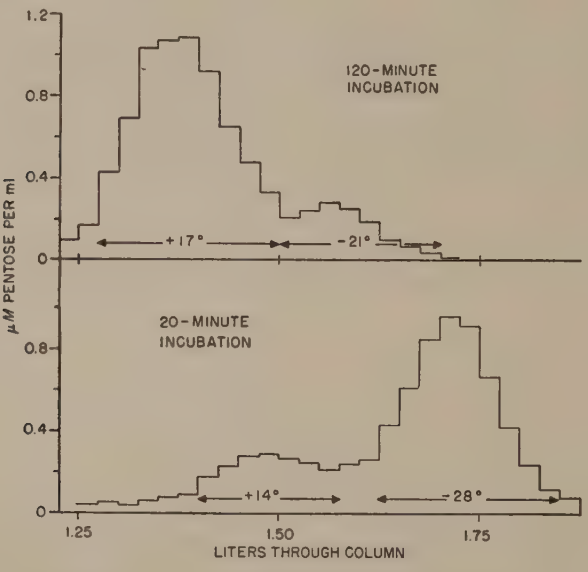


Fig. 3 The separation of pentose phosphates by ion-exchange chromatography.

5-phosphate. The other component which was levorotatory was obviously a precursor of ribose-5-phosphate, since it was the major component early in the reaction and was replaced by ribose-5-phosphate as the incubation was allowed to continue. This product was isolated directly from a 10-minute reaction mixture and hydrolyzed with potato phosphatase. The pentose was identified by color reactions and by the preparation of the crystalline *o*-nitrophenylhydrazone as D-ribulose, the 2-keto sugar corresponding to D-ribose. The other pentose ester was isolated from a two-hour incubation mixture

and, after enzymatic dephosphorylation, the identification of D-ribose was confirmed by the preparation of the benzylphenylhydrazone.

The purified phosphogluconic dehydrogenase preparation contains a pentose phosphate isomerase which catalyzes the interconversion of ribulose-5-phosphate and ribose-5-phosphate. After incubation for one to two hours an equilibrium mixture is formed which contains about 80% of the

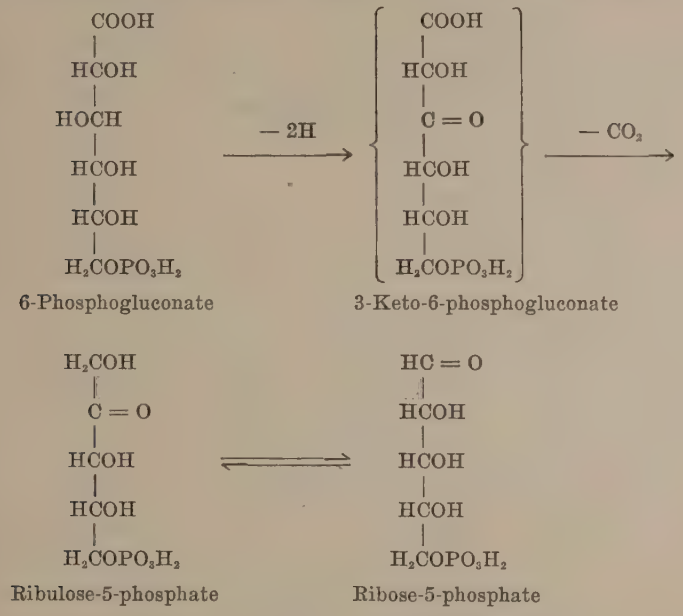


Fig. 4 The oxidation of 6-phosphogluconate.

aldose ester and 20% of the keto form. This equilibrium can be approached from either direction.

On the basis of these results we have written the scheme for the oxidation of 6-phosphogluconate which is shown in figure 4. To account for the formation of ribulose-5-phosphate as the primary reaction product, it might be assumed that oxidation of 6-phosphogluconate occurs at the β-carbon atom to form the hypothetical intermediate 3-keto-6-phosphogluconate. Decarboxylation of this β-keto acid would give ribulose-

5-phosphate which would then be converted by the action of pentose phosphate isomerase to ribose-5-phosphate. We have been unable to obtain direct evidence for the 6-carbon intermediate. There is no lag between oxidation and appearance of carbon dioxide which would be necessary for the accumulation of such an intermediate. During the early stages of the reaction when ribulose-5-phosphate predominates, there is an apparent lag or deficiency in pentose formation. This is due, however, to the low value given by ribulose-5-phosphate in the orcinol reaction. Compared with the usual arabinose or ribose standard, the color with ribulose-5-phosphate is 25–35% less intense. This error becomes negligible later in the reaction when only about 20% of the pentose is present as ribulose.

As we have written this reaction, it is an oxidative decarboxylation which resembles the conversion of malic acid to pyruvic acid and of isocitric acid to α-ketoglutaric acid (fig. 5). In each case a hydroxyl group in a β position to a carboxyl group is oxidized with the subsequent, or simultaneous, elimination of the carboxyl group. From the work from Ochoa's laboratory (Grafflin and Ochoa, '50; Salles, Harary, Banfi, and Ochoa, '50), it appears that the two steps, oxidation and decarboxylation, are catalyzed by single enzymes, the "malic enzyme" in the first case and isocitric dehydrogenase in the second case. The classical experiments of Ochoa and his group have demonstrated the fixation of carbon dioxide to occur by the reversal of these reactions. The similarity to the oxidative decarboxylation of malate and isocitrate suggested that the conversion of 6-phosphogluconate to ribulose-5-phosphate and carbon dioxide might also be reversible. In table 1 is shown the incorporation of radioactive carbon dioxide in phosphogluconate. This reaction was carried out in the presence of an excess of phosphogluconate and $C^{14}O_2$, but without the pyruvate system and with a limiting amount of TPN, so that only a small fraction of the phosphogluconate could undergo oxidation. Under these conditions about 1.5% of the total counts were fixed. Phosphogluconate, precipitated as the ba-

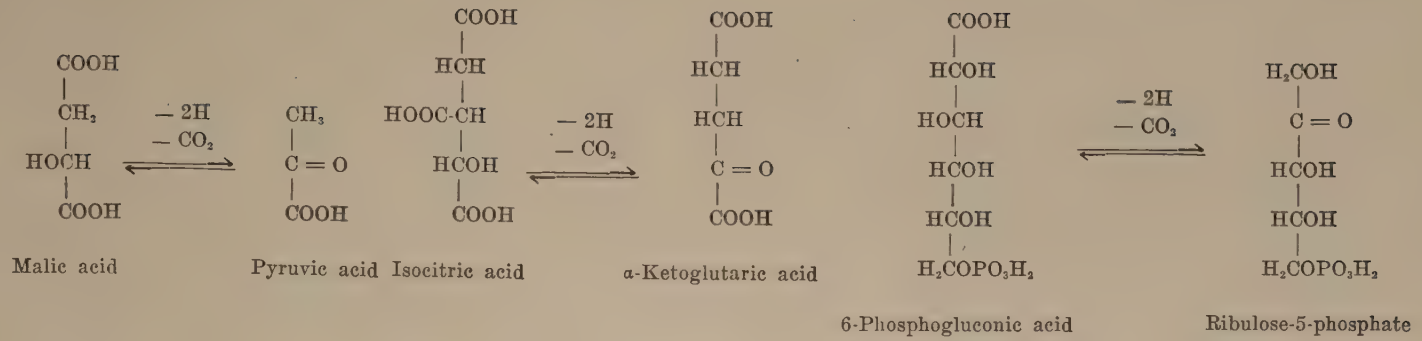


Fig. 5 Oxidative decarboxylation of β-hydroxy acids.

rium salt and converted to the sodium salt, contained practically all of the counts fixed. All the radioactivity was recovered as carbon dioxide when the phosphogluconate was oxidized with an excess of TPN.

This preliminary evidence for the reversibility of the reaction was supported by the spectrophotometric observation of the reoxidation of reduced TPN (fig. 6). In curve A, TPN was reduced with a limiting quantity of phosphogluconate, added at the time indicated by the first arrow. On addition of a bicarbonate-carbon dioxide solution at the second arrow very little reoxidation of TPNH occurred. If, however, an excess of ribulose-5-phosphate was present, as in curve B,

TABLE 1

Fixation of $C^{14}O_2$ in 6-phosphogluconate

	EXPERIMENT 1 [a]	EXPERIMENT 2 [b]
	cpm	*cpm*
Total $C^{14}O_2$ fixed	16,000	52,000
Recovered as barium salt	13,600	54,000
Recovered as $C^{14}O_2$ on enzymatic decarboxylation	12,700	52,500

[a] Incubated with 6 μM $C^{14}O_2$, 1,030,000 (cpm).
[b] Incubated with 22 μM $C^{14}O_2$, 3,600,000 (cpm).

then on addition of the bicarbonate-carbon dioxide mixture extensive reoxidation was observed. The effect of excess ribulose-5-phosphate in reducing the rate of TPN reduction by phosphogluconate, particularly in the later stages, was consistent with the reversibility of the reaction. The equilibrium constant could be calculated from the data in curve B, since all the concentrations were known from the amount of TPN reduced and the quantities of reactants added. This equilibrium constant was found to be 1.9 liters per mole, a value which is almost identical with the value of 1.3 reported by Ochoa ('51) for the oxidative decarboxylation of isocitrate. It means that in an atmosphere containing 5% of carbon dioxide and with half the TPN in the reduced form, over 99% of the phosphogluconate would be oxidized. In spite of its re-

versibility, therefore, the reaction can be used for the quantitative determination of phosphogluconate. It should be noted that the same value for the equilibrium constant was obtained from the data in curve A, where no excess of ribulose-5-phosphate was added, with the concentration values calculated from the slight reoxidation of TPNH observed.

A net synthesis of 6-phosphogluconate from ribulose-5-phosphate and carbon dioxide was obtained by coupling the system to a reaction in which reduced TPN was generated.

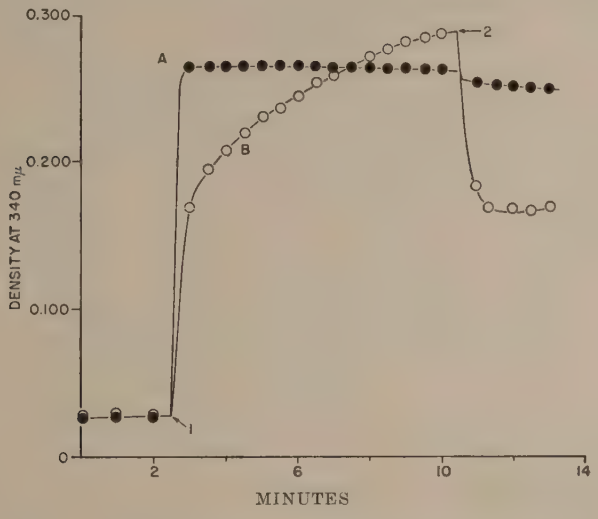


Fig. 6 The reversibility of 6-phosphogluconate oxidation.

The equations for this conversion are shown in figure 7. In this system, energy for the reductive carboxylation of ribulose-5-phosphate was provided by the oxidation of glucose-6-phosphate, which reacted with TPN to form 6-phosphogluconate and reduced TPN. As a result, 2 moles of 6-phosphogluconate should be formed for each mole of glucose-6-phosphate oxidized. One of these is formed by oxidation of the glucose-6-phosphate and the other arises by the reductive carboxylation of ribulose-5-phosphate. The results are shown in table 2 and are in agreement with this formulation. Glucose-6-phosphate and 6-phosphogluconate were assayed spectrophotometrically

with their respective dehydrogenases. In the complete system, as was expected, 2 moles of 6-phosphogluconate were formed for each mole of glucose-6-phosphate utilized. In the absence of pentose phosphate, a small amount of glucose-6-phosphate was oxidized by the TPN added, but no excess phosphogluconate was formed. No reaction occurred without glucose-6-phosphate. Under favorable conditions as much as 30% of pentose phosphate added could be converted to phosphogluconate.

So few reactions for the fixation of carbon dioxide are known that interest immediately arises in the possible role of this

Glucose-6-phosphate + TPN → 6-phosphogluconate + TPNH + H^+
Ribulose-5-phosphate + CO_2 + TPNH + H^+ → 6-phosphogluconate + TPN

Sum: Glucose-6-phosphate + ribulose-5-phosphate + CO_2 → 2,6-phosphogluconate

Fig. 7 Equations for the reductive carboxylation of ribulose-5-phosphate.

TABLE 2

Reductive carboxylation of ribulose-5-phosphate

	GLUCOSE-6-PHOSPHATE	6-PHOSPHOGLUCONATE
	μM	μM
Complete system	— 1.33	+ 2.58
Ribulose-5-phosphate omitted	— 0.30	+ 0.16
Glucose-6-phosphate omitted	0	— 0.01

reaction in photosynthesis. To obtain hexose by the fixation of carbon dioxide in phosphogluconate, however, would require that the oxidation of glucose-6-phosphate also be reversible. Two recent observations in the literature encouraged us to seek evidence for this reversibility. Strecker and Korkes ('51) had demonstrated the reduction of gluconolactone to glucose in the presence of glucose dehydrogenase from liver. Recently, Cori and Lipmann ('52) found that, in the oxidation of glucose-6-phosphate by *Zwischenferment,* the primary reaction product was the 6-phospho-δ-gluconolactone. On theoretical grounds the lactone should be much more readily reduced than the free carboxyl group. In table 3 are shown

the results of some experiments which establish the reversibility of glucose-6-phosphate oxidation. The δ-lactone of 6-phosphogluconate was prepared by the oxidation of glucose-6-phosphate with bromine. The amount of lactone present was determined by the hydroxamic acid reaction of Lipmann and Tuttle ('45). To provide reduced TPN for the reduction we used either phosphogluconate and phosphogluconic dehydrogenase or isocitrate and pig heart isocitric dehydrogenase. In either case about a third of the lactone originally present was reduced to glucose-6-phosphate, despite the fact that under

TABLE 3

Reduction of 6-phosphogluconolactone

REDUCING SYSTEM	LACTONE PRESENT INITIALLY	GLUCOSE-6-PHOSPHATE FORMED
	μM	*μM*
(1) + (3) 6-Phosphogluconate	0.70	0.23
(2) + (3) Isocitrate	0.76	0.23

(1) 6-Phosphogluconic acid + TPN $\xrightarrow{\text{phosphogluconic dehydrogenase}}$ ribulose-5-phosphate + CO_2 + TPNH + H^+

(2) D-Isocitric acid + TPN $\xrightarrow{\text{isocitric dehydrogenase}}$ α-ketoglutaric acid + CO_2 + TPNH + H^+

(3) 6-Phosphogluconolactone + TPNH + H^+ $\xrightarrow{\textit{Zwischenferment}}$ glucose-6-phosphate + TPN

the conditions of the experiment it was undergoing rapid hydrolysis to phosphogluconate. In control experiments with the γ-lactone, formed by heating phosphogluconate in acid, no reduction was observed. No glucose-6-phosphate was formed in the absence of TPN.

It has thus been demonstrated that, with the addition of energy in the form of reduced TPN, pentose phosphate and carbon dioxide can be reduced to form glucose-6-phosphate. The recent very interesting observations of Vishniac and Ochoa ('52) indicate that photosynthetic mechanisms may be able to provide the reduced coenzymes for these reactions.

Our next concern was with the further metabolism of pentose phosphate. With crude enzymes from liver and bone marrow, Seegmiller in our laboratory had observed that pentose phosphate formed from phosphogluconate was further metabolized and one of the products was identified as glucose-6-phosphate (Seegmiller and Horecker, '52). The extent of this reaction is shown in figure 8. With a crude ammonium sulfate fraction from rat liver there was a rapid disappearance of either ribulose- or ribose-5-phosphate and a corresponding

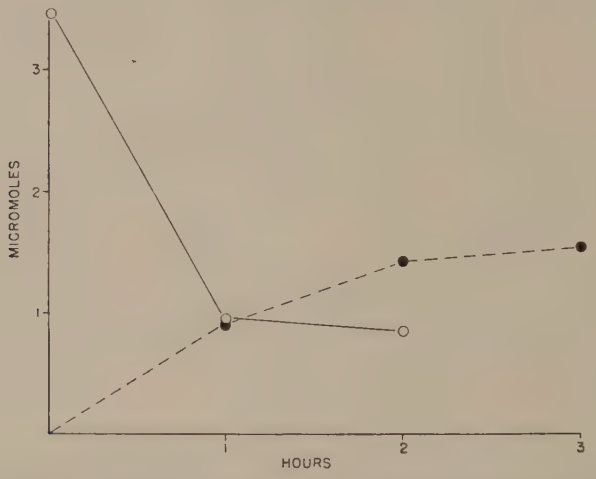


Fig. 8 Hexose phosphate formation from pentose phosphate with rat liver extracts.

– – – – ● = hexose-6-phosphate ———— ○ = pentose-5-phosphate

accumulation of a mixture of glucose- and fructose-6-phosphate. This was not a new finding. It had been reported a number of years ago by Dische ('38) and more recently by Waldvogel and Schlenk ('47). Taken together with the observation of Racker ('48) that pentose phosphate could be split to form triose phosphate, it seemed reasonable to account for the formation of hexose phosphate by the series of reactions shown in figure 9. According to this scheme, triose phosphate formed from pentose phosphate would condense to form hexose diphosphate which would be dephosphorylated by Gomori's phosphatase (Gomori, '43) to form

fructose-6-phosphate. Dische ('49) found, however, that added fructose diphosphate was not converted to hexose monophosphate with his red-cell preparations and this enzyme also proved to be absent in our rat-liver fractions. This ruled out fructose diphosphate as a precursor of hexose monophosphate in these preparations. All attempts to substitute other precursors, such as triose phosphate and glycolaldehyde, for pentose phosphate were negative and so we turned to a study of the pentose phosphate-splitting reaction.

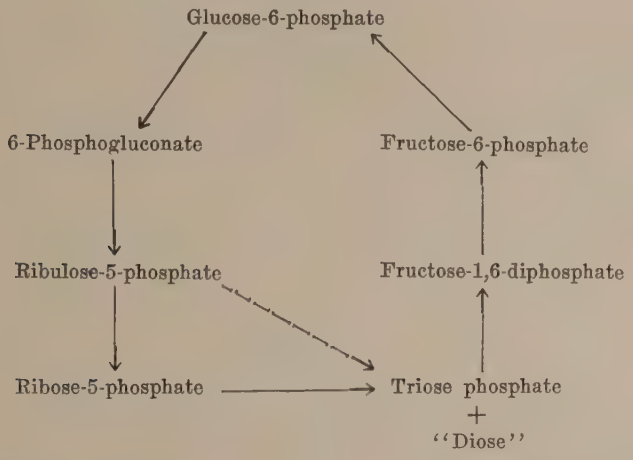


Fig. 9 Possible reactions in the formation of glucose-6-phosphate from pentose phosphate.

An assay system which could be used for the pentose phosphate-splitting enzyme had already been described by Racker ('47) (fig. 10). It employs a crude rabbit muscle fraction which contains glycerophosphate dehydrogenase and triose phosphate isomerase. Thus the formation of triose phosphate by the action of the splitting enzyme will lead to the oxidation of reduced diphosphopyridinenucleotide (DPN) whether the primary cleavage product be glyceraldehyde-3-phosphate or dioxyacetone phosphate. In the presence of excess glycerophosphate dehydrogenase the rate of reduced DPN oxidation will depend on the amount of splitting enzyme present.

With this assay system the enzyme was purified from rat-liver acetone powder extracts. The purification procedure is shown in table 4. Rat-liver acetone powder was extracted in the cold with dilute alkaline phosphate. The extract was fractionated with ammonium sulfate. The fraction which precipitated between 50 and 70% saturation was about twofold

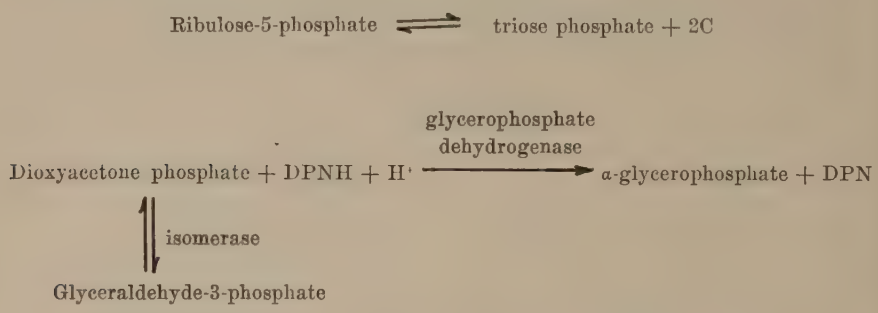


Fig. 10 Test system for the pentose phosphate-splitting enzyme.

TABLE 4

Purification of pentose-splitting enzyme from rat liver

FRACTION	UNITS	SPECIFIC ACTIVITY UNITS/MG
Acetone powder extract	667	0.031
Ammonium sulfate I	415	0.076
Methanol	330	0.44
Acetone	173	0.72
Ammonium sulfate II	130	1.65

purified. This was then fractionated with methanol at — 15°C. The activity precipitated between 52 and 65% methanol and was about sixfold purified. Some further purification was obtained by fractionation at — 7°C. with acetone between 31 and 43%. In the last step a fraction precipitating between 55 and 65% saturation with ammonium sulfate was collected. The final product was 50- to 70-fold purified compared to the original extract and the over-all yield was about 20%.

The purified preparation still contained pentose phosphate isomerase, so that it was active with either ribulose-5-phosphate or ribose-5-phosphate. However, while the crude extract attacked these substrates at equal rates, with the purified enzyme the rate was nearly twice as fast with ribulose phosphate as with ribose phosphate. On the basis of this observation it might be inferred that ribulose phosphate is the true substrate for the pentose phosphate-splitting enzyme.

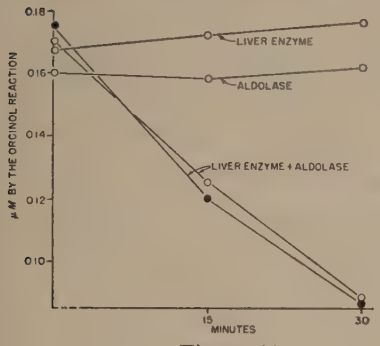


Figure 11

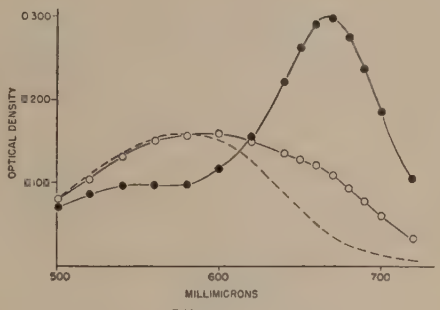


Figure 12

Fig. 11 The requirement of aldolase for pentose phosphate breakdown. Substrate: ○ = ribulose-5-phosphate ● = ribose-5-phosphate

Fig. 12 The product of ribulose-5-phosphate conversion with liver pentose-splitting enzyme and aldolase. Spectrum obtained in the orcinol reaction. ● ———— before incubation ○ ———— after 60 minutes' incubation – – – – – sedoheptulose

With the purified enzyme, an experiment was set up to accumulate the products of pentose phosphate splitting. However, in the absence of a mechanism for the removal of these products there was little disappearance of pentose phosphate (fig. 11). When the purified liver enzyme was incubated with ribulose-5-phosphate, the only change was a small increase in apparent pentose due to the conversion of some of the ribulose phosphate to ribose phosphate. If crystalline muscle aldolase (Taylor et al., '48) was also added, however, a rapid disappearance of pentose phosphate occurred. Aldolase alone was completely inactive. In the presence of both preparations ribulose-5-phosphate and ribose-5-phosphate disappeared at

about equal rates. These results suggested that the equilibrium for the splitting reaction favored the synthesis of pentose phosphate, and that it was necessary to remove a reaction product in order to bring about the utilization of pentose. The role of aldolase would be to catalyze a condensation of the cleavage products. This hypothesis was supported by an examination of the spectra obtained in the orcinol reaction (fig. 12). By the combined action of the pentose-splitting enzyme and aldolase, a new product was formed which reacted with orcinol to give a band at about 600 mμ, which replaced the band due to pentose at 670 mμ. The spec-

TABLE 5

Paper chromatography of heptuloses

(10 vol. acetone; 3 vol. water)

SUGAR	R_f
Reaction product	0.35
Sedoheptulose [a] (altroheptulose)	0.36
Idoheptulose [a]	0.41
Guloheptulose [a]	0.36
Galaheptulose [a]	0.22
Glucoheptulose [a]	0.27
Mannoheptulose [a]	0.27

[a] Kindly furnished by Dr. N. K. Richtmyer.

trum obtained was identical with that which would result from a mixture of heptulose phosphate and pentose phosphate. The absorption spectrum given by the 7-carbon sugar, sedoheptulose, is shown for comparison. Further evidence for the nature of this product was obtained by paper chromatography. Klevstrand and Nordal ('50) have recently described a spray reagent for heptuloses which has been shown by Bevenue and Williams ('51) to be highly specific for these compounds. The reagent is an orcinol-trichloroacetic acid (TCA) mixture which yields bright blue spots with heptuloses, yellow spots with ketohexoses, but no color with pentoses or other sugars. In table 5 is shown the result of a paper chromatogram sprayed with the orcinol-TCA reagent.

The chromatogram was developed with a mixture of 10 parts of acetone and three parts of water. The enzymically dephosphorylated reaction product is compared with a number of other heptuloses, including sedoheptulose (which is D-altroheptulose), idoheptulose, guloheptulose, galaheptulose, glucoheptulose, and mannoheptulose. While this solvent does not completely separate all the heptuloses, the reaction product behaved most like sedoheptulose or guloheptulose. It seemed most reasonable that it was the former, since this sugar occurs naturally.

The tentative identification of the reaction product as sedoheptulose was confirmed by the preparation of crystalline sedoheptulosan tetrabenzoate as described by Haskins et

TABLE 6

Sedoheptulosan tetrabenzoate

	MELTING POINT	OPTICAL ROTATION
	°C.	
Reaction product	164.5–165	—194°
Authentic derivative	164.5–165	—195°
Mixed	163.5–164	

al. ('52). The properties of this derivative are shown in table 6. The melting point of our derivative was 164.5–165°C., the same as that of the tetrabenzoate prepared from authentic sedoheptulosan. The mixed melting point was not significantly depressed. Both the authentic derivative and the enzymatic product had the same optical rotation.

The stoichiometry of heptulose phosphate formation from pentose phosphate is shown in table 7. Pentose phosphate and heptulose phosphate were determined with the orcinol reaction (Mejbaum, '39) by measurement of the absorption at two wave lengths, 670 and 580 mμ. Arabinose and sedoheptulose were used as standards and the concentration of the two sugars calculated by the solution of simultaneous equations. It was found that 2 moles of pentose phosphate disappeared

for each mole of sedoheptulose phosphate formed. This accounted for 7 of the 10 carbon atoms and should have resulted in the accumulation of 1 mole of a 3-carbon compound. Triose phosphate was indeed found to be present but, as

TABLE 7

Triose phosphate formation from ribose phosphate

	MICROMOLES	
	30 min.	60 min.
Pentose phosphate	— 3.59	— 4.78
Sedoheptulose phosphate	+ 1.54	+ 2.43
Triose phosphate (enzyme assay)	+ 0.91	+ 1.14
Triose phosphate (alkali-labile P)	+ 0.74	+ 0.71

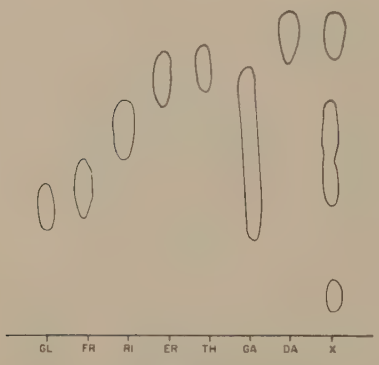


Figure 13

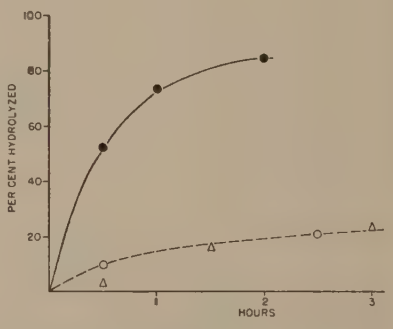


Figure 14

Fig. 13 Paper chromatogram of dephosphorylated reaction products (X) compared with glucose (GL), fructose (FR), ribose (RI), erythrose (ER), threose (TH), glyceraldehyde (GA), and dioxyacetone (DA).

Fig. 14 Acid lability of sedoheptulose phosphates, heated in 1 *N* H_2SO_4 at 100°C.

———— from HDP and erythrose – – – – – from pentose phosphate

assayed with the glycerophosphate dehydrogenase system, amounted to only about one-half the expected value. Since this assay system picks up hexose diphosphate as well as both triose phosphates, no explanation for the low recovery is available. The discrepancy between alkali-labile phosphate and triose phosphate by enzymatic assay is probably due to

the presence of some hexose diphosphate in the reaction mixture.

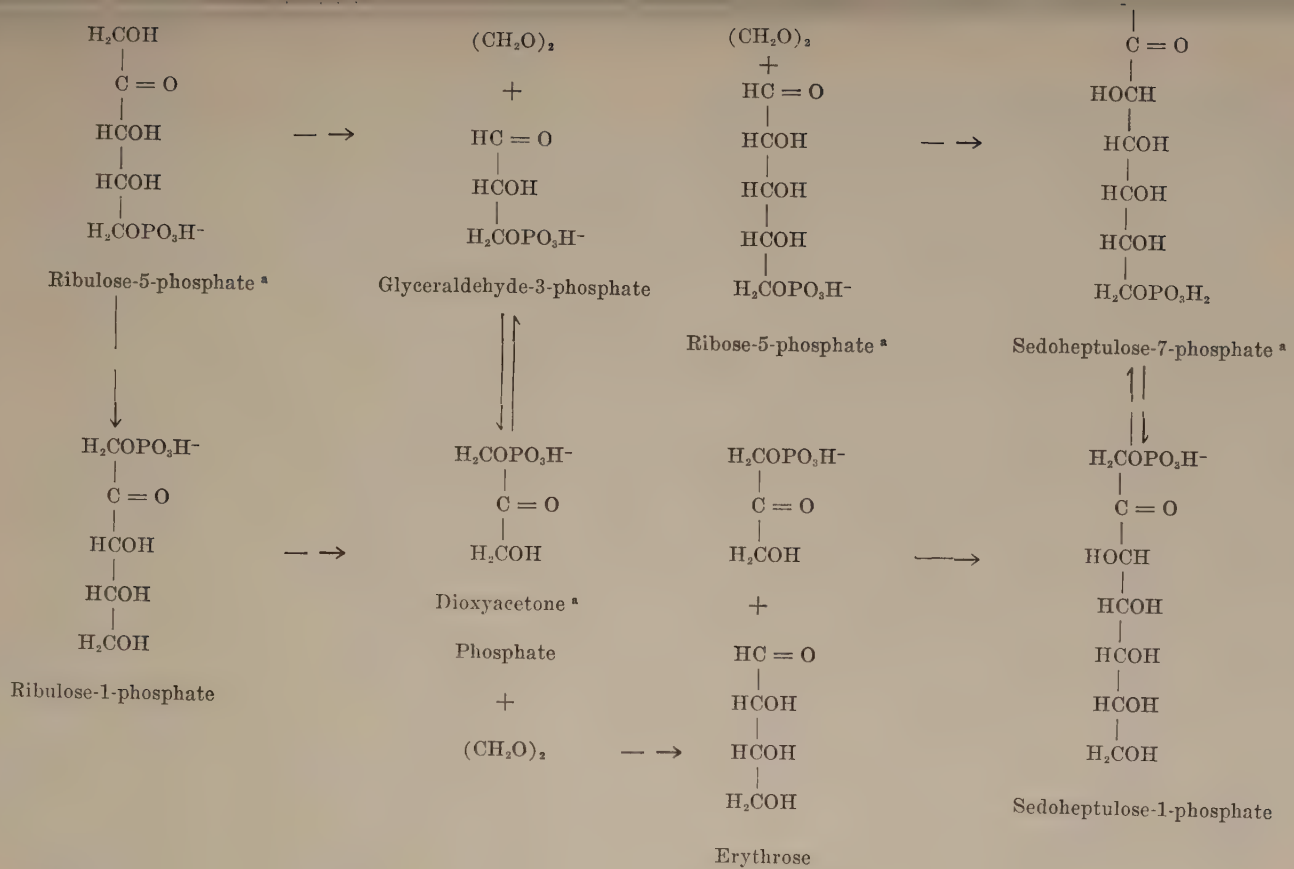
Paper chromatography of the dephosphorylated reaction products confirmed the presence of dioxyacetone. This is shown in figure 13. With the acetone-water solvent mixture developed in our laboratory by Seegmiller (Seegmiller and Horecker, '52) there is a distinct correlation between the number of carbon atoms and the R_f values. The hexoses, glucose and fructose, are slowest. Ribose moves a little faster. Ribulose, which is not shown, would be just ahead of ribose. The tetroses, erythrose and threose, are next and finally comes dioxyacetone. Glyceraldehyde occupies an anomalous position because of its tendency to form a dimer. In the products of the enzymatic reaction we see spots for sedoheptulose (with the smallest R_f value), some residual ribose, and dioxyacetone. There is also evidence for fructose, which is consistent with the previous indication for hexose diphosphate. While dioxyacetone and tetrose, particularly threose, are not very different in their R_f values, they can readily be distinguished by their color under ultraviolet illumination. When exposed to ultraviolet, the spots for the tetroses fluoresce a bright yellow, whereas the dioxyacetone spot has a brick-red fluorescence.

The sedoheptulose phosphate from the reaction mixture has been purified by ion-exchange chromatography. The ratio of sedoheptulose to phosphate in this product was 1:1. We are, therefore, dealing with a monophosphate ester. This ester is very resistant to acid hydrolysis, which is consistent with the structure, sedoheptulose-7-phosphate (fig. 14). A second ester having the properties of heptulose monophosphate has been obtained by the action of aldolase on hexose diphosphate and D-erythrose. This heptulose ester would, presumably, be formed by the condensation of dioxyacetone with the tetrose. From its lability to acid hydrolysis this product appears to be esterified in the 1 position. The sugar has not yet been identified, but on the basis of the *trans* configuration expected in condensations catalyzed by aldolase (Meyer-

hof et al., '36a, b), it is presumably sedoheptulose. We are indebted to Dr. Horace S. Isbell for the D-erythrose for these experiments.

With respect to the mechanism of formation of sedoheptulose phosphate in our system little information is yet available. Several alternative mechanisms can be written (fig. 15). Ribulose-5-phosphate might be split to form a 2-carbon fragment and phosphoglyceraldehyde, which would be converted to dioxyacetone phosphate. Sedoheptulose-7-phosphate might arise by a condensation of ribose-5-phosphate and the 2-carbon fragment. It is also possible that cleavage of the pentose phosphate occurs after migration of the phosphate group to the 1 position. This would directly yield dioxyacetone phosphate and again a 2-carbon fragment. Two such fragments could condense to form tetrose which would in turn condense with dioxyacetone phosphate to produce heptulose-1-phosphate. A mutase would be required to convert this to heptulose-7-phosphate. While such a mechanism is consistent with the observed condensation of D-erythrose with triose phosphate from hexose diphosphate, the condensation of the 2-carbon fragments would be expected to yield the *trans* rather than the *cis* configuration, and the final product would have the configuration of D-idoheptulose, rather than sedoheptulose, which is shown here. The nature of the 2-carbon fragment is of particular interest. None of the reactions observed can be reproduced with glycolaldehyde, which would be the 2-carbon compound expected to result from pentose phosphate cleavage.

Evidence for the nature of the 2-carbon fragment, as well as for the mechanism of erythrose formation, is provided by a recent report of Akabori et al. ('52). They found that dihydroxymaleic acid was decarboxylated by rabbit muscle mince and that 2 moles of the product would condense in an acetoin-like condensation to form erythrose. The mechanism suggested is shown in figure 16. Two moles of hydroxypyruvic acid would condense to form a ketotetrose which would be isomerized to erythrose. The latter was identified



[a] Identified in the reaction mixture.

Fig. 15 Possible reactions for sedoheptulose formation.

and evidence presented for the presence of isomerase. A subsequent condensation of erythrose with hydroxypyruvic acid led to the appearance of fructose and glucose, which were also identified. Ribose phosphate was formed by the condensation of dihydroxymaleic acid with triose phosphate. These condensations did not occur with glycolaldehyde. These results strongly suggest that the 2-carbon fragment formed from pentose phosphate is not free glycolaldehyde, but a bound form which can also arise from dihydroxymaleic acid or hydroxypyruvic acid.

To return briefly to sedoheptulose, this sugar was discovered in 1917 in the Sedum plant by La Forge and Hudson ('17). It has not previously been described in animal sources.

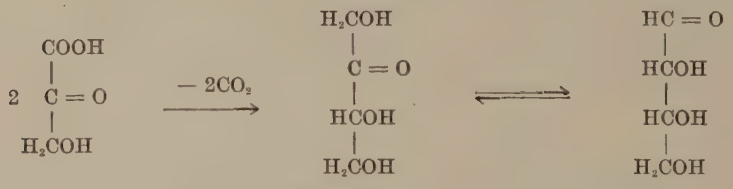


Fig. 16 Scheme for erythrose formation (Akabori et al., '52).

Recently, Benson et al. ('51) have observed two sedoheptulose phosphate esters among the early products of photosynthesis. They have suggested that these esters might play a vital role in the formation of hexoses. In this connection we have made some preliminary observations which are of interest. During the conversion of pentose phosphate to hexose phosphate in crude liver preparations, there is an early accumulation of sedoheptulose phosphate. This phase is followed by one in which the carbon atoms of sedoheptulose phosphate are converted by a yet unknown mechanism to hexose phosphate. These experiments suggest that sedoheptulose phosphate is an intermediate in the formation of hexose phosphate from pentose phosphate, and thus plays an important part in the metabolism of carbohydrates in animal tissues as well as in plants.

DISCUSSION

Chairman McElroy: Have you been able to study the problem of the 7-carbon sugar and its conversion to the 6-carbon sugar as yet?

Horecker: No. When we isolate the 7-carbon sugar from the mixture as the barium salt, convert it to the potassium salt, and return it to the crude liver preparations, we should expect it to go to hexose monophosphate. Unfortunately, this does not occur. We may be able to account for this negative result by a recent observation that magnesium ion added to the crude mixture seems to block completely the conversion of heptulose phosphate formed in the mixture to hexose phosphate. It may be that traces of barium which came through in our heptulose phosphate preparation were effective inhibitors. There is no question that the carbon atoms from the pentose which pile up in sedoheptulose must somehow be converted to hexose. There is no other precursor present. The stoichiometry is such that you cannot account for the carbon atoms which appear in hexose except by a formation from heptulose. This does not mean that the heptulose does not go back to pentose which is then converted to hexose by an entirely independent mechanism.

van Niel: Inasmuch as you have tried glycolaldehyde and found it ineffective in producing the product to be expected, you have concluded that a compound of this substance or something like it might not be involved. Would you consider it at all possible that this "bound glycolaldehyde" could be a slightly different sort of molecule; for instance, glycolic acid or glyoxylic acid?

In that event, obviously the first compound would not be a carbohydrate, but it could be a carbohydrate acid which by reduction could be converted to carbohydrate just as you have found that glycolic acid can be reduced.

Horecker: One difficulty with such an explanation is that our preparations carry out no oxidations or reductions. From pentose phosphate, when triose phosphate is removed, one is left with something at the oxidation level of glycolaldehyde,

and there is no way for this to be oxidized. This system which converts pentose phosphate to hexose phosphate is an anaerobic one. The only oxidative steps are those from hexose phosphate through ribulose phosphate.

VAN NIEL: In a sense, the over-all picture that I was trying to visualize would not involve any over-all oxidation or reduction.

HORECKER: Yes, but we have no evidence that something else is reduced to account for such oxidized products. The hydroxypyruvic acid which Akabori and his group postulated as an intermediate would, on decarboxylation, yield glycolaldehyde. Since glycolaldehyde itself is inert, it appears that hydroxypyruvate must undergo some condensation presumably to form an additional product, perhaps with the enzyme, which then loses carbon dioxide and gives a glycolaldehyde-enzyme or coenzyme compound which can undergo these condensations.

VAN NIEL: You see, you have to account for the formation of the 3-carbon compound from what one expects to come out as a 2-carbon fragment; and I do not see that this is any more difficult, nor any easier, than to account for the formation of a 2-carbon fragment that is not glycolaldehyde, but which *is* the corresponding acid.

HORECKER: Hydroxypyruvate may arise by transamination from serine which would in turn be formed by the addition of a 1-carbon compound to glycine. One fate of glycolaldehyde is a conversion back to glycine. A mechanism similar to this could account for the formation of a doubly labeled 2-carbon compound from a labeled 1-carbon precursor.

VAN NIEL: But still you would have to start with something like glycine or its carbohydrate equivalent.

HORECKER: Yes, one which contains no isotopic carbon dioxide.

VAN NIEL: My reason for being interested in a possible "active form" of glycolaldehyde is based on observations made by E. H. Anderson in 1941 in which he showed that an alga much like *Chlorella,* but which has no chlorophyll, responds in

a very peculiar manner to the addition of glycolic and glyoxylic acids. From his observations it appears that these two acids may well play a role in a cyclic mechanism.

HORECKER: They may be intermediates between glycolaldehyde and glycine.

LAMPEN: On this same point, I might mention experiments we did some years ago on pentose degradation in thymus extracts. We could readily obtain extracts which degraded the pentose phosphates and could account for the 3-carbon fragment as hexose diphosphate and monophosphates. I would suspect that we had fructose diphosphate as an intermediate, since these preparations would form inorganic phosphate from fructose diphosphate, and the inorganic phosphate formation was quite closely equivalent to the hexose monophosphate formation. Of course the two mechanisms would not be at all incompatible. Aldolase was present in our preparations and might have favored fructose disphosphate formation. The important thing was that, in these extracts, we could never account for any sizable amount of 2-carbon compounds as a single product. We could obtain small amounts of acetaldehyde, of bisulfite-binding compounds that were not phosphorylated, of material that acted like ethylene glycol, and so on. This would indicate that the 2-carbon compound disappeared very rapidly after formation. When glycolaldehyde was added to these extracts, it was inert. We were unable to demonstrate its oxidation or reduction with DPN, DPNH or TPN. We could obtain no evidence for free glycolaldehyde as an intermediate.

I might also mention that in work with intact cells of *L. pentosus* we obtained organisms which converted xylose or ribose to acetic and lactic acids, yet these organisms did not convert glycolaldehyde to acetic acid despite the fact that the 2-carbon unit, whatever it is, eventually ends up as acetic acid.

I think these findings are in agreement with the idea of a bound 2-carbon fragment.

GREEN: It may be of interest to point out that the pyruvic oxidase of animal tissues can oxidize hydroxypyruvate almost as rapidly as pyruvate. In the absence of an oxidizing agent, the oxidase decarboxylates pyruvate to a C_2 fragment, two of which condense to form an acyloin. In the case of hydroxypyruvate this acyloin would be a tetrose.

It is quite likely that the factor which the Japanese workers found capable of catalyzing tetrose formation may be pyruvic oxidase.

LIPMANN: While listening to Doctor Horecker's report that heptulose is generated from pentose, it occurred to me as an attractive possibility that two pentoses could condense to a 10-carbon chain, which then might break apart into 7- and 3-carbon chains. I wonder if such a reaction has been considered.

HORECKER: There is certainly no evidence to eliminate this possibility. On the other hand, we have none which would suggest it.

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ALTERNATE PATHWAYS OF HEXOSE OXIDATION IN PSEUDOMONAS FLUORESCENS

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TEN FIGURES

As a result of extensive research with yeast and bacteria, microbial glycolysis, in its over-all aspects, is reasonably well understood. In contrast to this, the oxidative utilization of glucose by aerobic bacteria has received little attention and is consequently less well understood. At present, several pathways of glucose oxidation are under consideration.

(1) The glycolytic conversion to pyruvate followed by its oxidation via the tricarboxylic acid cycle.
(2) The stepwise oxidation of glucose-6-phosphate through the hexose monophosphate pathway as described in yeast by Warburg and Christian ('31), Warburg et al. ('35), Dickens ('38a, b), Horecker et al. ('51), and Horecker and Smyrniotis ('51), and in *Escherichia coli* by Scott ('51).
(3) The stepwise oxidation of nonphosphorylated compounds involving gluconate and 2-ketogluconate as intermediates.

Various obligately aerobic bacteria among the closely related pseudomonas and acetobacter genera have been reported to follow the last pathway involving the non-phosphorylated intermediates. The finding of Barron and Friedemann ('41) that glucose oxidation by *Pseudomonas aeruginosa* was not inhibited by fluoride first indicated the existence of an oxidative pathway other than one involving the glycolytic system. Warburton, Eagles, and Campbell ('51) have demon-

strated the formation of pyruvate from glucose by a fluoride-insensitive mechanism, further suggesting the presence of another pathway. Studies of *Pseudomonas fluorescens* and *P. aeruginosa* by Lockwood et al. ('41), Lockwood and Stodola ('46), Stokes and Campbell ('51), and Entner and Stanier ('51) have shown that growing cultures, resting cells, and dried-cell preparations oxidize glucose to gluconate and 2-ketogluconate.

The pathway of 2-ketogluconate oxidation has not been clarified. Lockwood and Stodola ('46) and Koepsell ('50) have, however, demonstrated the formation of α-ketoglutarate and pyruvate from 2-ketogluconate by growing cultures. These reactions have not been defined in detail, possibly owing to the difficulty of obtaining dried-cell preparations or cell-free extracts which oxidize 2-ketogluconate.

Phosphorylated intermediates in the oxidation of glucose and gluconate to 2-ketogluconate by *P. aeruginosa* were excluded by Stokes and Campbell ('51). Neither the accumulation of phosphorylated intermediates during growth on glucose, nor the dependence of the glucose oxidation rate by dried cells upon adenosinetriphosphate (ATP) concentration could be demonstrated — nor was the oxidation inhibited by sodium fluoride. Thus, if the available data are to be interpreted rigorously, one is led to the conclusion that the pathway of glucose oxidation at least in its initial steps differs from that found in yeast and *E. coli*, namely, that the oxidation proceeds via nonphosphorylated intermediates.

Our observation that dried cells of *P. fluorescens* contain aldolase has suggested the possibility that additional pathways for hexose oxidation (i.e., the glycolytic system) may be present in this organism. Accordingly, further experiments have been undertaken to elucidate the steps in the pathways involving phosphorylated intermediates and to establish the role of these reactions in the oxidation of glucose.

For these experiments *P. fluorescens*, A 3.12, used in simultaneous adaptation experiments by Stanier ('47, '48) was grown in a mineral medium containing glucose. Cell-free

extracts, prepared by sonic oscillation and centrifugation of 7000 rpm yielded an opalescent, reddish extract with approximately 35 mg of protein per milliliter. Such extracts contained an intact electron transport system, since a wide variety of substrates were oxidized rapidly without the addition of hydrogen carriers such as phenazine derivatives or methylene blue.

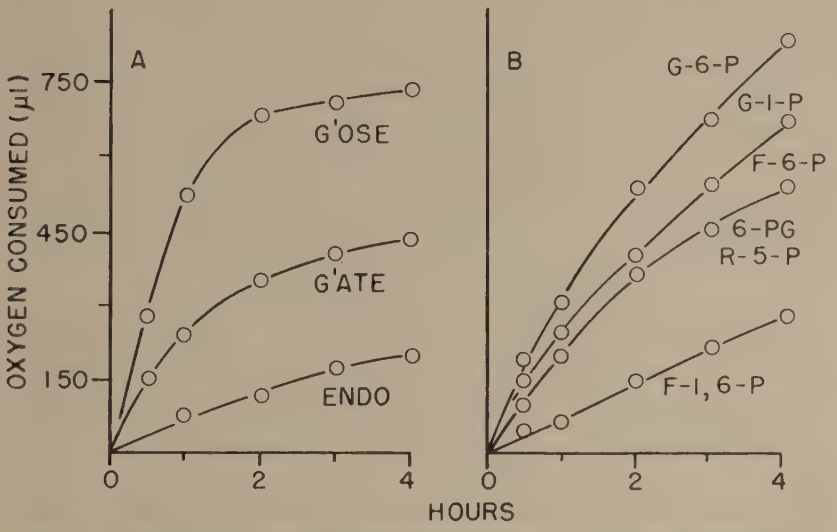

Fig. 1 Oxidation of various substrates by a sonic extract of *P. fluorescens*. Warburg cups contained approximately 35 mg of protein, 20 μM of substrate, and 100 μg of DPN (80% purity). G'ose = glucose; G'ate = gluconate; Endo = endogenous; G-6-P = glucose-6-phosphate; G-1-P = glucose-1-phosphate; F-6-P = fructose-6-phosphate; 6-PG = 6-phosphogluconate; R-5-P = ribose-5-phosphate; F-1,6-P = fructose-1,6-phosphate.

In order to obtain evidence as to possible routes of carbohydrate oxidation, the activity of the extract on a number of possible intermediates was tested (fig. 1). Oxygen uptake with glucose and gluconate proceeded rapidly, but soon slowed down to the endogenous rate. The net oxygen uptake approximated 2 atoms per mole of glucose and 1 atom per mole of gluconate, whereas 2-ketogluconate, D-ribose, and D- or L-arabinose were not oxidized. The extracts oxidized glucose-1-phosphate, glucose-6-phosphate, 6-phosphogluconate,

and ribose-5-phosphate. Fructose-6-phosphate also was oxidized, but the rate with fructose-1,6-diphosphate was only slightly above that of the endogenous. These observations cannot be interpreted in the light of any single pathway. It is, however, unlikely that glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were oxidized by the glycolytic pathway since the rate with hexose diphosphate oxidation was very low.

The rapid oxidation of glucose-6-phosphate and 6-phosphogluconate raises the question of the pathway of glucose and gluconate oxidation proceeding via these phosphorylated analogs. Since the phosphorylated forms are at the same oxidation state, similar oxygen uptake values with glucose and glucose-6-phosphate, and with gluconate and 6-phosphogluconate should be expected if a common pathway were involved. The following observations, however, are incompatible with such an interpretation. As shown in figure 2, the amount of oxygen taken up with glucose-6-phosphate and 6-phosphogluconate greatly exceeded the final amount obtained with glucose and gluconate. In addition, no carbon dioxide was produced in veronal buffer during the oxidation of glucose and gluconate, while more than 1 mole per mole was produced on glucose-6-phosphate and 6-phosphogluconate.

Preparations have also been obtained which oxidize glucose and gluconate, but not glucose-6-phosphate or 6-phosphogluconate (fig. 3). Either precipitation of the proteins with ammonium sulfate at pH 5 or the addition of neutral phosphate buffer to the sonic extract abolished the oxidation of glucose-6-phosphate and 6-phosphogluconate while the oxidation of glucose and gluconate was unaffected. Again 2 and 1 atoms of oxygen were taken up per mole of glucose and gluconate. Following the oxidation, the cup contents contained reducing material which was identified chromatographically by the method of Norris and Campbell ('49) as 2-ketogluconate. The chromatograms are illustrated in figure 4.

Using an ethanol-methanol-water system and an ammoniacal silver nitrate spray, glucose migrated with an R_f of

approximately 0.5 while gluconate and 2-ketogluconate occupied similar locations at R_f 0.24 to 0.27. After treatment with phenylhydrazine, the glucose and 2-ketogluconate spots disappeared, owing to the phenylhydrazone formation. No spot was found after chromatography of the reaction mix-

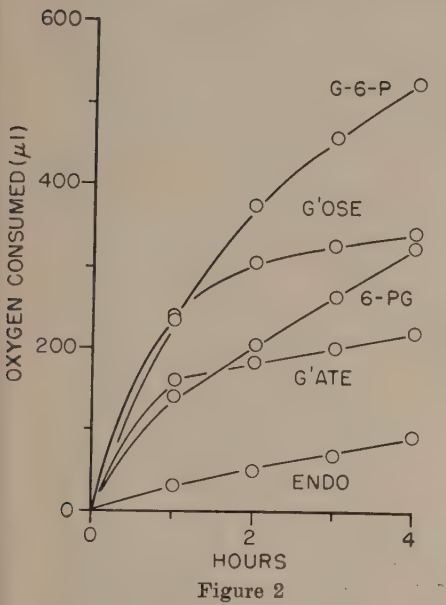

Figure 2

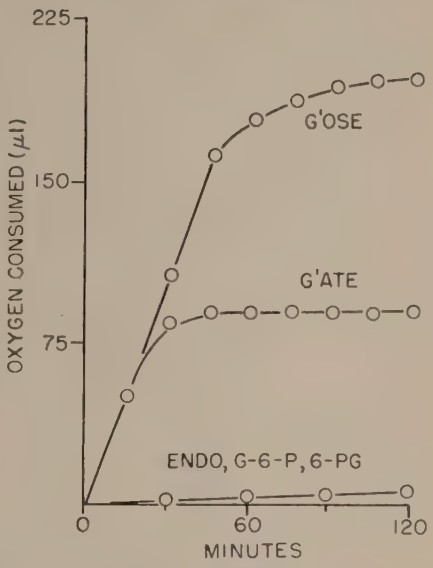

Figure 3

Fig. 2 A comparison of oxygen uptake on glucose and gluconate and the phosphorylated analogs. Conditions as in figure 1, except 10 μM of substrate were used. (Abbreviations as in figure 1.)

Fig. 3 Glucose and gluconate oxidation in the absence of glucose-6-phosphate and 6-phosphogluconate oxidation. A sonic extract was either precipitated at 75% ammonium sulfate staturation, pH 5, and tested in veronal buffer, pH 7, or tested without ammonium sulfate treatment in $M/10$ phosphate buffer, pH 7. Other conditions were as in figure 2. (Abbreviations as in figure 1.)

ture without substrate. From the cup containing glucose as substrate, no glucose spot was found after oxidation was complete, but a spot corresponding to gluconate or 2-ketogluconate was visible. A similar result was obtained with gluconate as a substrate. Following phenylhydrazine treatment, no silver spots were observed. This is characteristic of 2-ketogluconate. 5-Ketogluconate could not be eliminated

as the end product by this means. However, Entner and Stanier ('51) have established that 2-ketogluconate rather than 5-ketogluconate is the product of glucose oxidation by this organism.

The presence of an intact electron transport system in the crude extract, as evidenced by the rapid substrate oxidation without added carriers, prompted an investigation of the

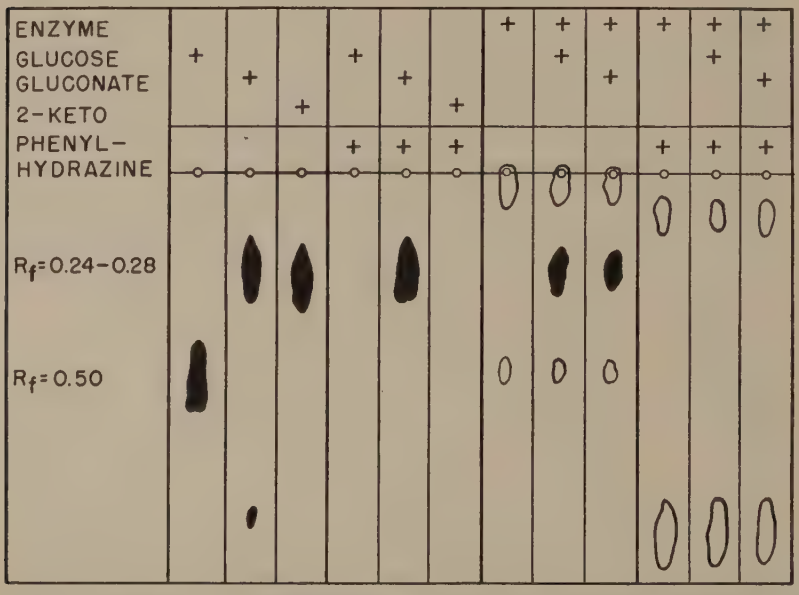

Fig. 4 Formation of 2-ketogluconate by glucose and gluconate oxidation. Cup contents following oxidation described in figure 3 were spotted on Whatman No. 1 paper, irrigated with a solvent mixture (40% ethanol, 40% methanol, and 10% water), sprayed with ammoniacal silver nitrate, and heated. A duplicate run was made after treating an aliquot with phenylhydrazine (10 mg/ml) at room temperature overnight (Norris and Campbell, '49).

nature of this system. Since catalase was present in the extracts, 1 atom of oxygen per electron pair would be consumed by either a flavoprotein or a cytochrome pathway. In the presence of cyanide, however, oxygen consumption by the flavin system would increase approaching 2 atoms per electron pair due to catalase inhibition, while the oxygen uptake by cytochrome carriers would be inhibited in the presence of

cyanide. As shown in figure 5, 10^{-3} *M* potassium cyanide strongly inhibited glucose and glucose-6-phosphate oxidation. The fact that complete inhibition was not achieved may indicate a slow rate of oxidation by flavoprotein carriers.

Glucose-6-phosphate and 6-phosphogluconate dehydrogenases were studied in more detail by measuring the rate of pyridine nucleotide reduction spectrophotometrically. Typical reduction curves are shown in figure 6. The rate of tri-

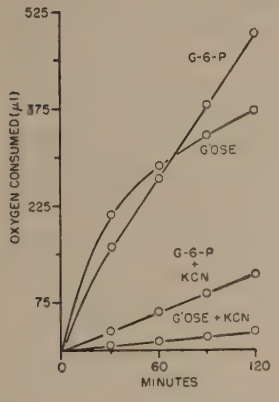

Figure 5

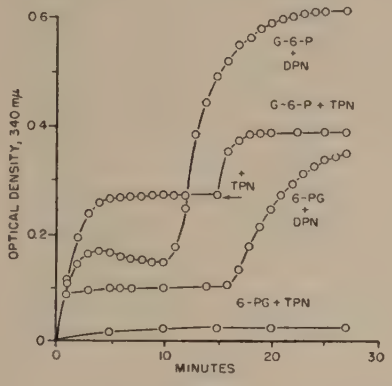

Figure 6

Fig. 5 Inhibition of oxygen uptake by cyanide. Conditions as in figure 1 except both neutralized potassium cyanide, 10^{-3} *M*, in main compartment and potassium hydroxide-potassium cyanide mixture in the center well where indicated.

Fig. 6 Reduction of DPN and TPN by glucose-6-phosphate and 6-phosphogluconate. Cuvette vol. = 3 ml containing 20 μM of substrate, 200 μg of TPN (43% purity 0.116 μM) or 250 μg of DPN (80% purity 0.3 μM), and 10^{-2} *M* trihydroxymethylaminomethane buffer, pH 7.

phosphopyridinenucleotide (TPN) reduction by glucose-6-phosphate was rapid, and the addition of more TPN resulted in a corresponding increase in density. Rapid diphosphopyridinenucleotide (DPN) reduction also was observed. In this case, however, the reaction was interrupted by a lag which was followed by a return to the maximal rate. This phenomenon was consistently observed, although the duration of lag varied in different preparations. A similar behavior was observed for DPN reduction by 6-phosphogluconate. In this preparation, TPN reduction by 6-phosphogluconate did

not occur. Coenzyme reduction by 6-phosphogluconate was not always reproducible in that preparations have been obtained which were inactive, while others reduced DPN, or both DPN and TPN. Ribose-5-phosphate, glucose-1-phosphate, and fructose-6-phosphate also served as hydrogen donors, whereas fructose-1,6-diphosphate, glucose, gluconate, and 2-ketogluconate were inactive.

The anomalous S-shaped DPN reduction curves may reflect the simultaneous reoxidation of reduced DPN (DPNH) by endogenous hydrogen acceptors. This reoxidation reaction could produce the S-shaped reduction curve as follows: During the initial period, DPNH is rapidly produced, and, as the concentration of DPNH increases, the rate of the reoxidation increases to a point where the reduction and reoxidation rates are equal. During this period, the net DPNH concentration does not increase and the plateau is observed. After the hydrogen acceptor has been utilized, the DPNH then accumulates. The plausibility of such a mechanism is supported by the fact that added DPNH was rapidly oxidized in the absence of substrate, and following this, no lag occurred during reduction by glucose-6-phosphate. However, neither dialysis nor fractionation removed the lag, thereby suggesting that organic acceptors such as pyruvate were not involved. To test the possibility that dissolved oxygen acted as the acceptor, the cuvettes were aerated at intervals. The results of this treatment and the corresponding changes in DPNH concentration are shown in figure 7. After the reduction of DPN had reached a maximum, aeration caused a rapid decrease in optical density followed by an approximately equal increase, indicating DPN oxidation followed by reduction. After cyanide addition, aeration no longer caused a decrease in DPNH concentration. With limiting substrate levels, the rapid oxidation of DPNH was more evident. Again the addition of cyanide inhibited the reoxidation and allowed the DPNH to accumulate. It appears that reduced DPN is reoxidized by oxygen with the electron transport mediated by cytochrome type of carriers. No re-

oxidation of reduced TPN was observed either in the presence or absence of cyanide. Thus it may be that the electron transport from reduced TPN to oxygen can occur only via

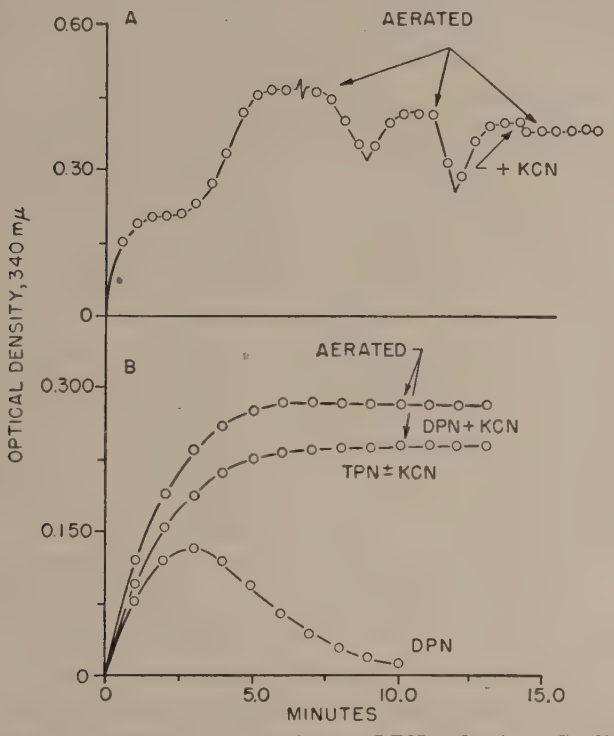

Fig. 7 Effect of aeration and cyanide on DPN reduction. Conditions as in figure 6 except 0.15 μM of glucose-6-phosphate used as the limiting substrate concentration (lower graph). In the upper graph, final concentration of KCN was $10^{-3} M$; the cuvette was aerated at points indicated by arrows (excess glucose-6-phosphate). DPN was added initially. In the lower graph both the coenzyme and the cyanide were added initially and the cuvettes aerated at points indicated by arrows.

DPN, a reaction catalyzed by pyridine nucleotide transhydrogenase.

The basis for the lack of TPN specificity for glucose-6-phosphate oxidation has not been investigated intensively. The presence of a pyridine nucleotide transhydrogenase, as recently described by Colowick et al. ('52), would explain

the observations. This enzyme, obtained from another strain of *P. fluorescens* catalyzes the transfer of electrons from reduced TPN to DPN. In the presence of the transhydrogenase, TPN-specific enzymes also reduce DPN. The reaction appears not to be readily reversible, hence TPN is the obligatory primary acceptor, and a trace of TPN must be present for DPN reduction to occur. While DPN reduction in *Pseudomonas* extracts could occur by this mechanism, several observations are inconsistent with this hypothesis. No dependence of DPN reduction rate upon TPN concentration has been observed either with a crude enzyme or with a fractionated preparation. In addition, a glucose-6-phosphate dehydrogenase fraction has been obtained which reduced DPN rapidly, but was inactive with TPN.

Dickens ('38a, b), and more recently Horecker et al. ('51) and Horecker and Smyrniotis ('51) have shown that in yeast extracts 6-phosphogluconate is oxidatively decarboxylated to pentose phosphate. The observation that *Pseudomonas* extracts catalyze oxygen uptake and pyridine nucleotide reduction in the presence of 6-phosphogluconate is superficially consistent with the observations made with yeast, and suggests that pentose phosphate is an intermediate in 6-phosphogluconate oxidation. However, with preparations which metabolized 6-phosphogluconate more rapidly than ribose-5-phosphate and ribulose-5-phosphate, only traces of pentose phosphate were formed during 6-phosphogluconate breakdown. This suggested the presence of another pathway for 6-phosphogluconate metabolism.

Recently Entner and Doudoroff ('52) have observed the formation of carboxyl-labeled pyruvate from 1-C^{14}-glucose and 1-C^{14}-gluconate by *Pseudomonas saccharophila*. The specific activity of the pyruvate was one-half that of the glucose used, indicating the formation of an equal amount of unlabeled pyruvate. In addition, cell-free extracts of this organism formed pyruvate and triose phosphate from 6-phosphogluconate.

With extracts of *P. fluorescens*, the disappearance of 6-phosphogluconate in the presence of sodium arsenite (5×10^{-3} *M*) and hydrazine (5.6×10^{-2} *M*) was accompanied by the formation of both pyruvate and alkali labile phosphate. The stoichiometry of the reaction is shown in table 1. With utilization of 8.2 μ*M* of the initial 12.4 μ*M* of 6-phosphogluconate, slightly less than an equivalent amount of pyruvate and alkali labile phosphate was formed. The deficiency in the pyruvate found can be accounted for by increase in pentose phosphate, thus accounting for virtually all the carbon. Under the same conditions, 7.7 μ*M* of 23 μ*M* of ribose-5-phosphate disappeared. However, neither pyruvate nor alkali labile phosphate accu-

TABLE 1

Stoichiometry of 6-phosphogluconate metabolism
(Pseudomonas fluorescens extract)

SUBSTRATE (μ*M*)	△ μ*M* — 30 MINUTES			
	6-Phosphogluconate	Pyruvate	OH⁻ Labile P	Pentose
None	..	0	..	+ 0.08
6-Phosphogluconate, 12.5	— 8.20	+ 7.40	+ 6.22	+ 0.84
Ribose-5-phosphate, 23	..	+ 0.1	0	— 7.7

mulated. Thus ribose-5-phosphate was not an intermediate in pyruvate and triose phosphate formation from 6-phosphogluconate. In a similar manner, ribulose-5-phosphate disappearance was not accompanied by pyruvate or triose phosphate formation. The finding of Horecker and Symrniotis ('52) that pentose phosphate utilization by a partially purified pentose-splitting enzyme from yeast was accompanied by sedoheptulose phosphate formation prompted a search for this compound as a product of pentose phosphate utilization by *P. fluorescens*. The disappearance of ribose-5-phosphate was accompanied by the appearance of a broad peak at 580–590 mμ in the orcinol determination for pentose similar to that reported by Horecker ('52) for sedoheptulose. The reaction mixture chromatographed on paper gave a blue spot

in the color test of Bevenue and Williams ('51) which is specific for ketoheptoses. While this compound has not been further characterized, it seems probable that sedoheptulose phosphate also is produced from pentose phosphate by *P. fluorescens*.

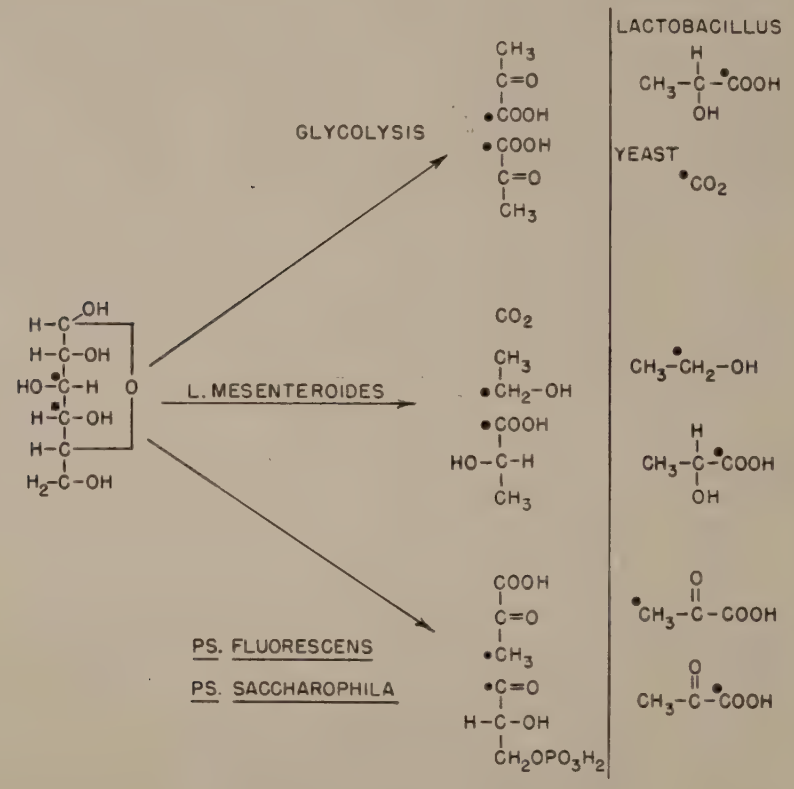

Fig. 8 Distribution of glucose carbons-3 and -4.

Entner and Doudoroff's demonstrations that the radioactivity from 1-labeled glucose is found in the carboxyl carbon of pyruvate indicates the existence of a third pathway for forming 3- and 2-carbon units from carbohydrate. These pathways are shown in figure 8. In the glycolytic scheme, carbons-3 and -4, shown by the dots, become the carboxyl carbons of pyruvate, and form either the carboxyls of lactate

in the homolactic fermentation by lactic acid bacteria or the carbon dioxide in the ethanol fermentation by yeasts. In the *Leuconostoc mesenteroides* fermentation, studied by Gunsalus and Gibbs ('52), carbon-3 became the carbinol carbon of ethanol and the carbon-4 the carboxyl carbon of lactate. In *P. saccharophila* and *P. fluorescens,* carbon-3 becomes the methyl carbon of pyruvate and carbon-4, the carbonyl carbon of triose phosphate. Since Doudoroff has shown the oxidative formation of 2 moles of pyruvate from 1 mole of glucose and the conversion of triose phosphate to pyruvate in *P. saccharophila,* it is evident that carbon-3 becomes the methyl carbon of one pyruvate, and carbon-4 is the carboxyl carbon of the other.

Extracts of *P. fluorescens* prepared by sonic oscillation have been separated into two components either by high speed centrifugation or by precipitation with ammonium sulfate. The fraction obtained as a gel by centrifugation or by precipitation with neutral ammonium sulfate at 33% saturation appeared to be particulate in nature. Stanier (personal communication), who has studied this fraction, has obtained the janus green reaction which has been reported by Mudd and co-workers ('51) to be characteristic of bacterial mitochondria. The reddish particulate fraction containing the cytochrome system rapidly oxidized glucose-6-phosphate and glucose, but oxidized gluconate slowly. The addition of a soluble protein fraction obtained between 33 and 66% ammonium sulfate saturation increased the rate of gluconate oxidation by the particles.

The distribution of dehydrogenases in the two fractions is shown in figure 9. Glucose-6-phosphate dehydrogenase which reduced either DPN or TPN was present in the particulate material and was absent from the soluble fraction. The reduction of DPN by 6-phosphogluconate was catalyzed exclusively by the soluble fraction. It is of interest to note that the lag in DPN reduction due to reoxidation by oxygen was absent, probably due to removal of the cytochrome system with the particles. Again TPN was not reduced.

By using a longer period of sonic oscillation, it has been possible to obtain a portion of the glucose-6-phosphate dehydrogenase activity in soluble form. After removal of the particles and precipitation of the soluble proteins, this fraction reduced DPN rapidly. TPN was completely inactive. In the crude material or particle fraction the rates on DPN and TPN were approximately equal. Thus it appears that DPN reduction by glucose-6-phosphate is catalyzed by a DPN-specific *Zwischenferment.*

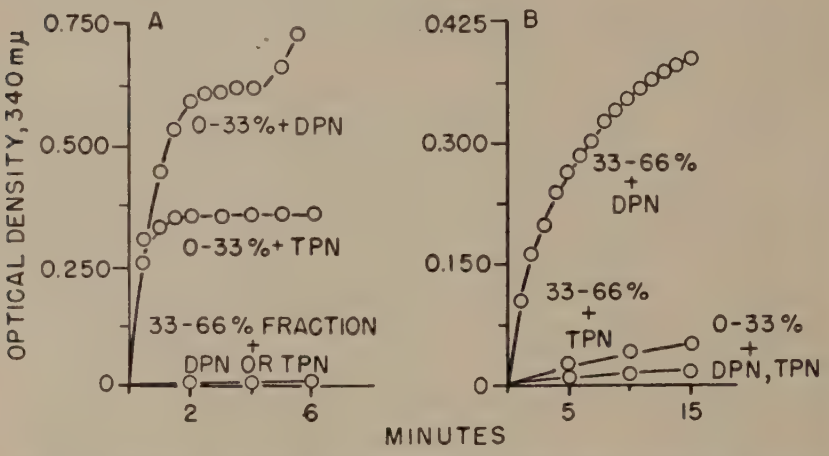

Fig. 9 Pyridine nucleotide reduction by ammonium sulfate fractions. Particulate material precipitated by adding 0.5 volume of neutral saturated ammonium sulfate. Soluble proteins obtained by adding 2.5 additional volumes to supernatant. Fractions dissolved in water. A. Substrate, glucose-6-phosphate. B. Substrate, 6-phosphogluconate.

The pathways of hexose oxidation in *P. fluorescens* extracts are summarized in figure 10. Glucose is oxidized to gluconate and 2-ketogluconate. All the evidence indicates that the reaction proceeds independently of the phosphorylated intermediates. Several attempts to demonstrate hexokinase activity in dried cell preparations and sonic extracts have been negative. Glucose-6-phosphate is oxidized presumably to 6-phosphogluconate with DPN or TPN as the coenzyme. 6-Phosphogluconate is split largely to pyruvate and triose

phosphate. A small amount of pentose phosphate is formed. Pentose phosphate is metabolized, but triose phosphate and pyruvate did not accumulate. The presence of phosphohexose isomerase, aldolase, and phosphoglucomutase has been demonstrated. It is attractive to speculate on the presence of a cycle for glucose-6-phosphate oxidation, involving 6-phosphogluconate, and triose phosphate, two triose phosphates forming hexose diphosphate, and finally glucose-6-phosphate again.

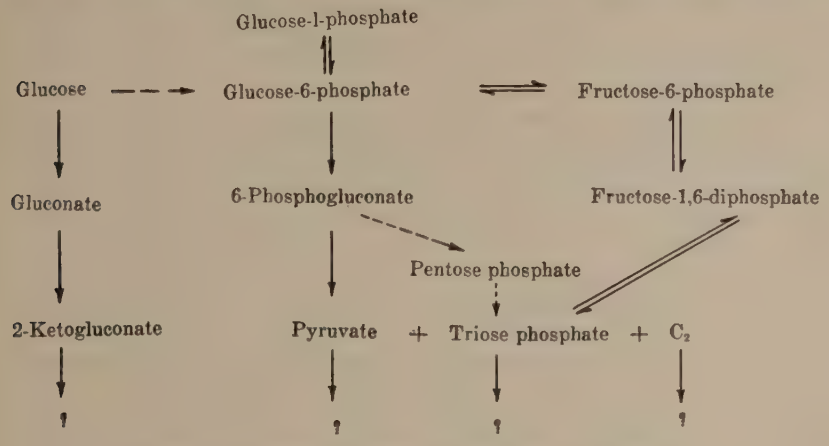

Fig. 10 Oxidative Scheme, *Pseudomonas fluorescens.*

However, the slow rate of hexose diphosphate oxidation eliminates such a possibility as being the main oxidative pathway for glucose-6-phosphate oxidation.

DISCUSSION

KAPLAN: In connection with the sonic extracts which respire, are these clear extracts?

W. A. WOOD: For oxygen uptake studies, we centrifuge the sonic extracts at approximately 7000 rpm. This removes the cellular debris, what little there is, but leaves a lot of material in solution and the solution is not very clear. After centrifugation at higher speeds a gel is obtained, as I mentioned, which is probably particulate in nature; but we have never been able to remove these particles — if that is what they are

— completely from the extract by centrifugation. That is why we resort to ammonium sulfate fractionation. By this means the particles can be precipitated completely.

KAPLAN: I might mention that we have noted respiration in clear extracts of *Pseudomonas,* but we always had to add a carrier such as one of the chloroindophenol dyes. There is what appears to be a diaphorase in these clear *Pseudomonas* extracts.

The most interesting thing that appears is an oxidative enzyme which is sensitive to cyanide and relatively insensitive to azide.

This enzyme does not appear to be a cytochrome oxidase; it does not oxidize reduced cytochrome c, nor does it require cytochrome c for activity, and the enzyme appears to have a high turnover number. We are now engaged in its purification.

W. A. WOOD: In the experiments outlined, pyruvate was determined first enzymically with lactic dehydrogenase, and then eventually with 2,4-dinitrophenylhydrazine reagent after finding that the two methods agreed. The question arises here, in connection with the mechanism of pyruvate formation from 6-phosphogluconate, whether or not hydroxypyruvate might not be the split product. Does hydroxypyruvate behave as pyruvate, using an enzymatic assay with lactic dehydrogenase? Certainly it does with the 2,4-dinitrophenylhydrazine reagent.

GREEN: I do not recall whether the reduction of hydroxypyruvate by the lactic dehydrogenase can be tested for directly. But there is some indirect evidence that the lactic dehydrogenase can act upon hydroxypyruvate.

KALLIO: The answer to Dr. Wood's question, I suspect, is that it is still open. I do not know, however, that Dr. Doudoroff actually got two pyruvates when he measured them as the 2,4-dinitrophenol hydrazones. Yet, on chromatograming these 2,4-dinitrophenol hydrazones, there were two spots. I think, on the basis of this, that question is still open.

W. A. Wood: Was the other spot the methyl glyoxyl?

Kallio: No.

Lipmann: You have no evidence for your glyceric acid formation?

W. A. Wood: No. Dr. Doudoroff thought of this as a possibility but was unable to show the conversion of glyceric acid to pyruvate. We have not tested this.

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PENTOSE AND DESOXYPENTOSE METABOLISM IN BACTERIA [1]

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EIGHT FIGURES

OXIDATION OF PENTOSES

Bertrand (1898) was the first to observe oxidation of pentoses by the acetic acid bacteria. In his experiments, xylonic acid was formed in amounts which accounted quantitatively for the disappearance of xylose. Thus the pentonic acid had not been oxidized. Since that time many reports of pentose oxidation have appeared but one generally cannot be sure that the pentonic acid was the sole product. For instance, although Hermann and Neuschul ('31) showed that the pentonic acid was the only acid product in their cultures, they did not estimate the residual pentose. Since the yield of acid was only about 50% in terms of the initial pentose, further oxidation might well have occurred.

Hayasida ('38) observed that certain *Fusaria* oxidized xylose rapidly to xylonic acid but acted on xylonic acid extremely slowly. More rapid oxidation of the acids was found by Higuchi et al. ('51). They observed that a strain of *Brucella melitensis* oxidized D-galactose and L-arabinose (these have the same configurations at carbons-2, -3, and -4 — fig. 1) to the corresponding acids. On further incubation the keto acids accumulated in moderate yields. Oxidation of the keto acids

[1] This paper is based in part on work performed for the Biological Department, Chemical Corps, Camp Detrick, Frederick, Maryland, under contracts nos. W-18-064-CM-241 and DA-18-064-CML-466 with Western Reserve University.

was slow. With D-glucose and D-xylose (these again have identical configurations at carbons-2, -3, and -4), there was a more rapid oxidation of the sugar, with a lesser accumulation of the keto acid, and apparently more rapid further oxidation.

The studies described thus far have yielded no evidence for the formation of phosphorylated products; however, this is

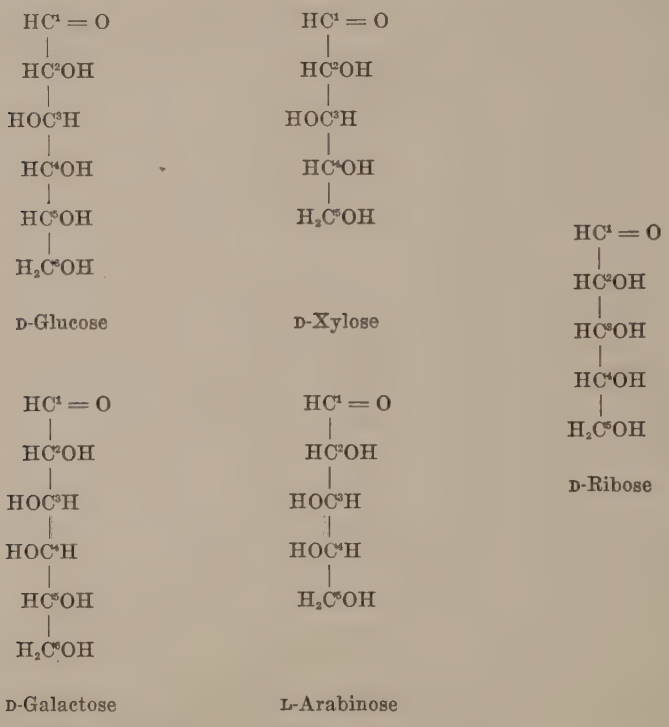

Fig. 1 Structures of related hexoses and pentoses.

still possible. The oxidation of ribose-5-phosphate by yeast has been studied by Dickens ('38a, b) and by Sable ('51); it is a complex process requiring at least two enzymes, with isomerization of the pentose phosphate probably necessary prior to oxidation. Cleavage of the carbon chain may occur as well. One comment should be added in discussing the potential step-wise oxidative degradation. Barker and Lipmann ('49) observed the phosphorylation of erythritol by propionic

acid bacteria. Acid was formed, but no carbon dioxide or phosphoglyceric acid could be demonstrated. These workers suggested oxidation of the initial erythritol phosphate to an erythrose phosphate and then to a tetronic acid phosphate. This is the type of conversion expected during step-wise degradation of a pentose.

FERMENTATION OF PENTOSES

The general mechanisms which have been proposed for pentose fermentation are outlined in figure 2. (The oxidative step-wise degradation is indicated at the top of the figure.) The most frequently suggested scheme has been cleavage of the

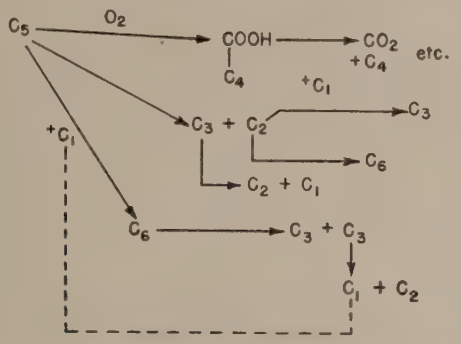

Fig. 2 Possible routes of pentose metabolism.

carbon chain into C_2 and C_3 units. This was supported primarily by the observation that a number of the lactic acid bacteria form equivalent amounts of acetic and lactic acids during the fermentation of pentoses (Fred et al., '19, '21). The question at present is whether or not this can be considered as the initial mechanism of cleavage in most fermentations with the variations in *end products* of pentose metabolism being the result of variations in the subsequent fate of the C_2 and C_3 units. Another suggested mechanism is that of addition of a C_1 unit to the C_5 chain to yield a hexose with subsequent cleavage of the hexose to two C_3 units. It should be mentioned that by this mechanism there is no net change

in the balance of end products of the fermentation from that expected with an initial C_2–C_3 cleavage. This is on the assumption that the C_1 unit is present only in catalytic amounts, as it must be in most fermentations. The scheme may be attractive, however, as a means of explaining the formation of specific fermentation products. Another possibility, really a variant of the C_2–C_3 cleavage, is that of secondary condensation of the C_2 unit, either with C_1 forming a C_3 unit, or with other C_2 fragments to C_6 units, which could then be cleaved to C_3 compounds.

Additional evidence has been obtained recently in favor of the C_2–C_3 cleavage with both the homofermentative and hetero-

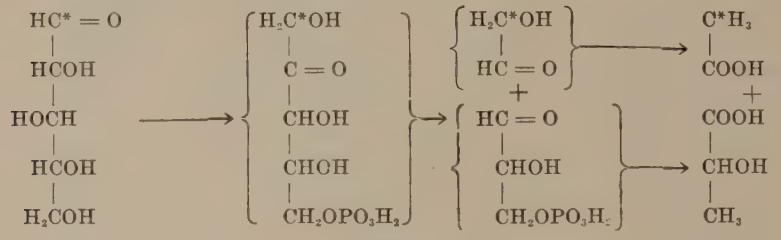

Fig. 3

fermentative lactic acid bacteria. We have studied the metabolism of 1-C^{14}-D-xylose by the homofermentative organism *Lactobacillus pentosus* (Lampen et al., '51; Gest and Lampen, '51). The labeled xylose was incubated with cells grown on xylose and the resulting acetic and lactic acids were isolated. Only the acetic acid contained significant amounts of C^{14} and essentially all the C^{14} was present in the methyl group (fig. 3). This showed that the methyl group of the acetic acid was derived from the aldehyde carbon of xylose. We presume then that carbon-2 of xylose is the precursor of the acetic acid carboxyl and that carbons-3, -4, and -5 are converted to lactic acid.[2] These results offer strong support of the hypothesis

[2] Dr. I. A. Bernstein recently obtained biologically labeled samples of ribose and has degraded them by fermentation with *L. pentosus* and by a chemical procedure. His results confirm our suggestions as to the fate of carbons-2 to -5 of the pentose chain. (Personal communication.)

of an initial cleavage of pentoses into C_2 and C_3 fragments and indicate that splitting occurs between carbons-2 and -3. If cleavage of the 1-C^{14}-xylose had occurred between carbons-2 and -3 by means of an "aldolase type" reaction, i.e., between carbons α and β to a carbonyl group, the C_2 compound formed would contain C^{14} in the -CHO group. One would thus expect the -COOH group of the final acetic acid to contain a large fraction of the C^{14}. Since this portion of the acetic acid was completely inactive, it is most reasonable to conclude that such a reaction did not occur. The observed distribution of activity can be explained readily if a 2-ketopentose is postulated as an intermediate in the fermentation. Cleavage of this ketopentose would yield a C_2 compound with the C^{14} in the more reduced group and this group might then be converted to the CH_3 group of the acetic acid. The formation of the phosphate esters is suggested on the basis of data to be presented subsequently. In the figure, glycoaldehyde has been indicated as the C_2 product of cleavage only to show which is the more reduced and which the more oxidized grouping. I should point out that the mechanism of the cleavage reaction suggested here is not novel. It is formally the reverse of that generally written for the formation of acetylmethylcarbinol from two molecules of acetaldehyde, that is, it is a reverse "acyloin" condensation.

Evidence for the occurrence of a similar cleavage in a heterofermentative lactic acid organism was obtained by Rappoport et al. ('51). They studied the fermentation of 1-C^{14}-L-arabinose by *Lactobacillus pentoaceticus,* and also observed that the aldehyde carbon of the sugar was the precursor of the CH_3 group of the acetic acid. This over-all mechanism of pentose fermentation appears then to hold for both the homofermentative and heterofermentative organisms.

Evidence for a C_2–C_3 cleavage has also been presented by Kaushal et al. ('51) who obtained glyoxyl 2,4-dinitrophenylosazone (presumed to arise from glycolaldehyde) from cultures of *Acetobacter acetigenum* growing on xylose or arabinose. They conclude that this isolation is proof of a

cleavage of the molecule into C_2 and C_3 units. However, this group of bacteria catalyzes rapid oxidation of pentoses and one would like to be certain that the glycolaldehyde had actually arisen from the carbons-1 and -2 of the pentose. Experiments with labeled sugars appear essential here.

Turning now to some of the evidence indicating more complex patterns of pentose degradation, one might consider the recent studies of Nutting and Carson ('52a, b) with *Escherichia coli*. At neutral or slightly alkaline pH this organism ferments xylose with the production of small quantities of lactic and succinic acids, and larger quantities of formic and acetic acids and of ethanol. As the pH was decreased, the quantity of lactic acid formed per mole of xylose increased, and at pH's less than about 5.8 it was in excess of 1 mole per mole of pentose. It is obvious that this could not have arisen by a simple C_2–C_3 cleavage of the chain. These workers observed that the high yields of lactic acid occurred under conditions where there was a net uptake of carbon dioxide; they suggested an initial cleavage of the pentose into C_2 and C_3 fragments with the C_2 fragment then coupling with a C_1 unit to yield lactate. They observed that when $C^{14}H_3COONa$ or $C^{14}H_3CH_2OH$ was added during xylose fermentation, the isolated lactate contained C^{14} in the β carbon but did not have any detectable C^{14} in the α carbon and only small amounts in the carboxyl group. Addition of $HC^{14}OOH$ or $C^{13}O_2$ resulted in incorporation of isotope into the carboxyl of the lactate. These results are not in agreement with formation of the lactate via a C_4 dicarboxylic acid, a tricarboxylic acid cycle, or a glycine-formate condensation. They can be explained most simply by the assumption that a direct $C_2 + C_1$ condensation has occurred. Unfortunately, such studies give no information on the nature of the C_2 compound actually involved, nor of the C_2 unit obtained from the pentose in this system.

The fermentation of hexoses and pentoses often results in very similar mixtures of products. The observations of Adams and Stanier ('45) are rather characteristic and table 1 con-

tains some of their data. It may be seen that the same end products are formed from the two sugars and in comparable quantities. There are obvious differences, however, such as that in the amounts of 2,3-butylene glycol. Also the quantity of carbon dioxide formed is considerably in excess of 1 mole per mole of pentose, as anticipated from the simplest form of the C_2–C_3 cleavage, where a C_1 molecule would arise only from the C_3 unit. These workers suggested that either addition of a C_1 unit had occurred initially to yield a C_6 compound which was metabolized as was the glucose, or that the C_2 unit, if formed, might be resynthesized to a C_6 unit. This would,

TABLE 1

Products of hexose and pentose fermentation by Aerobacillus polymyxa

PRODUCT	MOLES/100 MOLES OF SUGAR FERMENTED	
	Glucose	Xylose
2,3-Butylene glycol	65.1	38.0
Acetoin	2.8	2.5
Ethanol	66.2	63.0
Acetate	2.9	7.7
Carbon dioxide	199.6	161.0
Hydrogen	70.9	82.0

of course, also explain the occurrence of similar products from the two sugars.

In regard to the formation of more than one C_1 unit per mole of pentose, the oxidation-reduction relations of the hexose and pentose fermentations should be considered. This was pointed out most clearly, I believe, by Johnson et al. ('31) in studies on the acetone-butanol fermentation by *Clostridium acetobutylicum*. Here again several workers had observed the formation of 1.2–1.4 moles of carbon dioxide per mole of pentose and Van der Lek ('30) had suggested the initial formation of a C_6 molecule. As is shown in figure 4, if one writes a formal equation for the conversion of a hexose to 2 C_2 moles (at the carbohydrate oxidation level) and 2 moles of carbon dioxide, 8H must be written in to balance the equation. Thus

8H are available for the reduction of 2 C_2 units. In an analogous equation for a pentose, one obtains 2 C_2 units plus 1 mole of carbon dioxide and only 4H. It is obvious then, that the C_2 units and their products as obtained from the pentose fermentation will, on the average, be at a higher oxidation level than will those from a hexose fermentation. Instead of oxidation of the H available from the hexose, oxidation of some C_2 units will occur. It is probable that considerable amounts of carbon dioxide would be formed under these conditions. This offers a reasonable explanation of the high yields of carbon dioxide observed with pentoses and removes at least the necessity for postulating a more complex type of initial cleavage.

$$C_6H_{12}O_6 \rightarrow 2C_3 \rightarrow 2C_2H_4O_2 + 2CO_2 + 8H$$

$$C_5H_{10}O_5 \rightarrow C_2 + C_3 \rightarrow 2C_2H_4O_2 + CO_2 + 4H$$

$$\downarrow$$

$$C_1$$

Fig. 4

Two reports of *qualitative* differences in the products from pentoses and from hexoses appear of particular interest. One is that of Reynolds and Werkman ('37) who observed that a strain of *E. coli* produced carbon dioxide and hydrogen in good quantities during the anaerobic decomposition of glucose, but produced very little of these gases during xylose fermentation. The basis of this difference is obscure at the moment but may be related to the reported effect of glucose on hydrogenlyase production (Lascelles, '48). Another interesting report is that of Heald ('52) who described the fermentation products obtained with 5 "intermediate" strains of *E. coli* obtained from the rumen contents of sheep. These organisms were grown in a partially synthetic medium simulating the growth conditions in the rumen and with an atmosphere of carbon dioxide. Heald observed that glucose and cellibiose were fermented with the production of formic, ace-

tic, and lactic acids, of ethanol, and of the C_4 compounds acetoin and 2,3-butylene glycol. In the degradation of either xylose or glucuronic acid these C_4 compounds were not formed in detectable quantities. Unfortunately, Heald did not present complete carbon balances for these fermentations; in fact, in the experiments with xylose, only 20–30% of the carbon was recovered. Thus, we have no real idea of what variations occurred. Heald suggests that the lack of acetoin and butylene glycol as products of the pentose fermentation indicates the necessity of an essential catalytic intermediate formed only during growth on glucose. The fact that glucuronic acid gave a fermentation pattern similar to that from xylose is indirect evidence for its conversion to a pentose compound by removal of carbon-6.

Concerning the possible conversion of the C_2 unit to hexoses, it might be pointed out that a number of bacteria are able to grow on C_2 units and certainly synthesize considerable amounts of hexose. Kaushal and Walker ('51) have investigated one of the more readily accessible models of this reaction, the formation of cellulose from sugars by species of *Acetobacter*. They observed formation of cellulose from pentoses and from ethylene glycol. During growth on ethylene glycol it was possible to demonstrate accumulation of glycolaldehyde, which must be at least closely related to the C_2 compound formed from pentoses.

This brief survey of the literature on pentose fermentation indicates two well-documented paths of metabolism of the original C_5 chain. The first is characterized by oxidation of carbon-1 and may well involve step-wise degradation of the chain. In the second pathway something at least formally equivalent to a C_2–C_3 cleavage occurs. The products may then undergo a variety of metabolic alternations; i.e., the C_3 unit may undergo cleavage to C_2 and C_1 units, the C_2 unit may combine with a C_1 unit, C_2 or C_3 may in some manner condense to yield C_6 units, and finally the C_2 units may be oxidized to yield the excess of C_1 units observed in many pentose fermentations. It is obvious that one can only say such a sum-

mation is in agreement with the fermentation balances and isotopic data presently available. Further studies with isotopically labeled compounds will probably be necessary before the path of the individual carbon atoms of the sugars can be traced with assurance.

DEGRADATION OF DESOXYRIBOSE NUCLEOSIDES

Information on the fermentation of desoxyribose, the other important type of C_5 sugar, is relatively meager. The only

TABLE 2

Fermentation of thymidine by Escherichia coli

	VALUES CORRECTED FOR ENDOGENOUS
	μM
Desoxyribose disappearance	39
Products:	
Hydrogen	16.8
Carbon dioxide	16.5
Formate	11.3 (28.1[a])
Acetate	33.4
Ethanol	33.3
Propionate	1.2
Propanol	2.2
Carbon recovery	88%
Free thymine	35 (90%)

[a] Total "formate;" see text.

50 μM of thymidine were incubated in a Warburg flask with 250 mg (wet weight) of cells in 2.0 ml of 0.1 M phosphate buffer pH 7.2. Gas phase, nitrogen; sodium hydroxide was present in the center well.

balances available, to the author's knowledge, on fermentations of desoxyribose compounds are those performed in our laboratory (Hoffmann and Manson, '51; Hoffmann and Lampen, '52). Table 2 shows the data of a typical fermentation of thymidine by *E. coli* strain 15. The carbon dioxide and hydrogen are assumed to have arisen from formate, since these organisms contain an active hydrogenlyase. The balance represents essentially the conversion of 1 mole of desoxyribose to 1 mole each of formate, acetate, and ethanol, i.e., to 2 C_2 units and a C_1 unit. The carbon recovery in all such

experiments is about 85–90% and the recovery of free thymine is in the same range. The missing carbon is at the oxidation level of glucose and probably has been incorporated into cell substances. Racker ('51) demonstrated the formation of desoxyribose-5-phosphate from triose phosphate and acetaldehyde in extracts of *E. coli*. Our observation that two C_2 compounds and a C_1 compound were formed from the desoxyribose nucleoside is in agreement with this reaction and supports the idea of an initial cleavage of desoxyribose phosphates to a

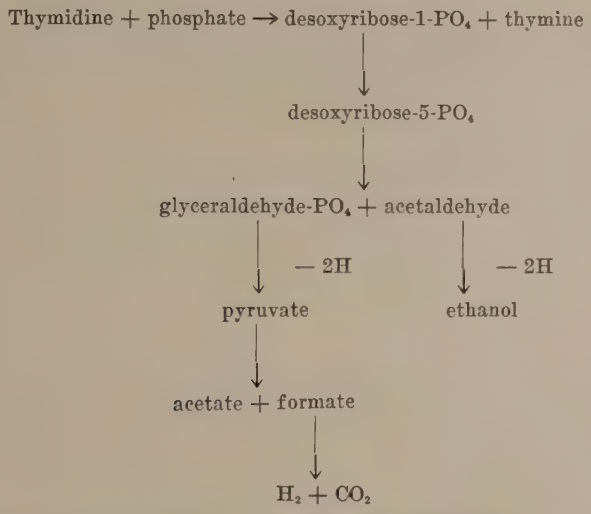

Fig. 5 Suggested pathway of thymidine degradation in *E. coli*.

triose phosphate and acetaldehyde. Thus in the one organism where desoxypentose degradation has been studied in some detail, a C_2–C_3 cleavage occurs with that (as with pentoses) occurring between carbons-2 and -3 of the chain. The probable pathway of thymidine fermentation is outlined in figure 5.

MECHANISM OF PENTOSE FERMENTATION BY LACTOBACILLUS PENTOSUS

The homofermentative organism *Lactobacillus pentosus* was chosen for these studies, since it appeared to offer the

simplest over-all system of pentose degradation. The experiments with 1-C^{14}-xylose indicated the formation of C_2 and C_3 units. It was also known that the organism would grow readily on D-ribose, D-xylose, or L-arabinose, but not on D-arabinose. The question which arose then was which of these pentose compounds actually underwent cleavage. Most specifically, we wondered if the individual pentoses were degraded by separate though similar routes, or whether the pathways fused, as is the case with many of the hexose sugars. As an approach to this question, we studied the specificity of pentose fermentation by cells raised on the various sugars (Lampen and Peterjohn, '51). We had noted that xylose fermentation

TABLE 3

Specificity of fermentation by Lactobacillus pentosus

CARBOHYDRATE IN MEDIUM	RATE OF SUBSEQUENT ACID PRODUCTION FROM			
	D-Glucose	D-Ribose	D-Xylose	L-Arabinose
D-Glucose	++++[a]	+	—	—
D-Ribose	++++	++++	—	+
D-Xylose	++++	++++	++++	+
L-Arabinose	++++	++++	—	++++

[a] Rates are graded from —, no fermentation, to ++++, maximum rate obtained with given cell type.

by washed cells of this organism required the presence of xylose in the growth medium, and it was felt that a more detailed study of this apparent "adaptation" might reveal some interrelation of the degradative pathways of the various pentoses. Cells were grown on a medium containing 1% of the proper sugar and the washed cells tested for activity in fermenting the various pentoses and glucose. The results obtained are summarized in table 3. Each cell type ferments rapidly both glucose and the sugar present in the growth medium. The interesting feature, however, was the activation of the ribose-fermenting system by growth in the presence of the other pentoses. Thus, growth on xylose or L-arabinose gave cells which fermented ribose rapidly, whereas growth on

ribose did not yield cells with significant activity on the other pentoses.

On the preliminary assumption that the biochemical basis of these alterations in specificity was that of alterations in the enzymic composition of the cells, an effort was made to demonstrate such differences. It was soon discovered that growth of the cells on any of the three pentoses resulted in cells with greatly increased activity in degrading ribose-5-phosphate. Data obtained with equivalent amounts of sonic

TABLE 4

Action of cell-free extracts of Lactobacillus pentosus on ribose and on pentose phosphates

CARBOHYDRATE IN MEDIUM	SUBSTRATE	μM OF PENTOSE AT INDICATED TIMES			
		0 hr.	0.5 hr.	2 hr.	5 hr.
Glucose	Ribose	8.8	9.1	8.5	..
	Ribose-5-phosphate	8.0	7.8	5.7	5.4
Xylose	Ribose	6.6	6.2	6.5	..
	Ribose-5-phosphate	7.2	2.8	2.3	2.2
	Xylose-5-phosphate	6.2	5.9	6.1	..

Each incubation mixture contained the substrate, 0.5 ml of the enzyme preparation (equivalent to 250 mg of wet cells) and 1.0 ml of 0.1 *M* Na-K phosphate buffer, pH 7.0. Final volume — 4.0 ml. At the indicated times samples were deproteinized by adding an equal volume of 4% perchloric acid. The protein-free filtrates were analyzed for pentose. All values have been corrected for the small changes observed in a parallel incubation without substrate.

extracts of glucose-grown and of xylose-grown cells are presented in table 4. Neither extract degraded free ribose (in the absence of added adenosinetriphosphate — ATP). Ribose-5-phosphate was degraded only slowly by the extract of cells grown on glucose but degradation was rapid with the extract of the cells from the xylose medium. It is interesting to note that the extract of the xylose-grown cells degraded ribose-5-phosphate but not xylose-5-phosphate. Similar data were obtained with extracts of cells grown on ribose or on L-arabinose. The D-arabinose-5-phosphate was not degraded by any of the extracts.

The data on ribose phosphate degradation and on fermentation specificity can probably be explained most simply by assuming that D-xylose and L-arabinose are converted to compounds with the ribose configuration (see fig. 1) by *L. pentosus* before cleavage of the pentose chain occurs. This would explain the correlation between growth of the cells in media containing pentoses and the increased activity of extracts prepared from these cells in degrading ribose-5-phosphate. Small amounts of free ribose might form during this process and bring about the observed "adaptation" to ribose. However, free ribose is probably not an essential intermediate in these conversions, since by storage of the washed cells in a refrigerator for several days, one can readily obtain cells whose rate of acid production from xylose is several times that with ribose. One would suggest, therefore, that "adaptation" to free ribose is not an essential feature of the suggested mechanism of xylose degradation.

With this hypothesis of the nature of the metabolic pathways of D-xylose and of L-arabinose as a guide, the initial reactions of pentoses in these organisms were examined. The extracts obtained by sonic disintegration catalyzed the phosphorylation of the pentoses by ATP. Characteristic results obtained with extracts of cells grown in a xylose medium are given in table 5. The D-xylose was phosphorylated rapidly, with the reaction essentially complete in 90 minutes. In the absence of ATP there was no decrease in free xylose. Glucose and ribose were phosphorylated at one-fourth and one-third the rate observed with xylose, whereas D- or L-arabinose and D-2-desoxyribose were not utilized at a significant rate.

The results obtained with the various extracts are summarized in a qualitative way in table 6. An extract of cells grown on glucose phosphorylated glucose rapidly, ribose slowly, and xylose and L-arabinose at negligible rates. Cells grown on ribose utilized ribose rapidly, glucose at a fair rate, and the other sugars little if at all. Xylose-grown cells gave an extract which phosphorylated xylose rapidly, ribose and glucose at moderate rates, and L-arabinose not at all. Lastly,

extracts of cells grown on L-arabinose phosphorylated arabinose at a moderate rate, glucose and ribose rapidly, but did not act on xylose. It is obvious that an excellent correlation exists between the rates of acid production from the various

TABLE 5

Phosphorylation by extracts of cells grown on xylose

SUGAR	AMOUNT OF SUGAR	μM PHOSPHORYLATED	
		40 min.	360 min.
	μM		
D-Glucose	5.6	0.8	2.2
D-Ribose	5.6	1.1	4.3
D-Xylose	6.0	3.4	5.8
D-Xylose [a]	6.0	0.1	0.0
D-Arabinose	5.6	— 0.3	0.4
L-Arabinose	5.9	0.1	— 0.5
D-2-Desoxyribose	3.2	0	0

[a] No ATP added.

Each incubation mixture contained 0.2 ml of the sonic extract (equivalent to 100 mg wet weight of original cells), 7.2 μM of ATP, 0.1 ml of 1 M sodium fluoride and the substrate in a total volume of 2.5 ml of 0.03 M tris-(hydroxymethyl)aminomethane buffer, pH 7.8. Aliquot samples were deproteinized with barium hydroxide-zinc sulfate at the indicated times and the filtrates analyzed for residual reducing sugar or pentose. Temperature 30°C.

TABLE 6

Specificity of phosphorylation by extracts of Lactobacillus pentosus

SUGAR IN GROWTH MEDIUM	PHOSPHORYLATION BY EXTRACT			
	D-Glucose	D-Ribose	D-Xylose	L-Arabinose
D-Glucose	++++	+	±	—
D-Ribose	+++	++++	±	—
D-Xylose	++	++	++++	—
L-Arabinose	++++	++++	±	++

sugars by the whole cells and the ability of extracts of these cells to catalyze the phosphorylation of the sugars. One may conclude, therefore, that one of the first steps in the metabolism of these sugars is a phosphorylation by ATP to yield phosphate esters.

We then attempted to demonstrate the formation of ribose phosphate during xylose metabolism. Two types of enzyme preparations have been used. The extracts for the first experiments were prepared by sonic disintegration in phosphate buffer of cells grown on xylose. With such preparations the rate of xylose phosphorylation is two to 4 times the rate of pentose phosphate degradation (compare tables 4 and 5), and the phosphate esters should accumulate. ATP was added in catalytic amounts and continuously regenerated from added phosphoglyceric acid with a dialyzed dog brain acetone powder. The balance for such an experiment is given in figure 6. The 23 μM of degraded pentose and 29 μM of accumulating pentose phosphate total 52 μM, as against the 55 μM of xylose phosphorylated. Most of the added ATP could be ac-

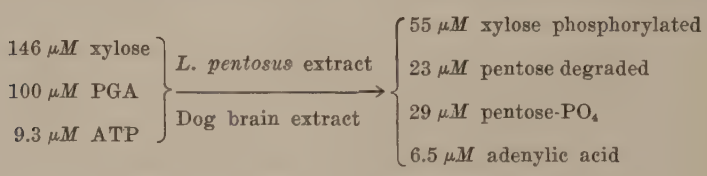

Fig. 6 Products of xylose phosphorylation.

counted for as adenylic acid, with small amounts of ADP (adenosinediphosphate) and ATP probably remaining as well, so that this ribose could not have made a significant contribution to the pentose phosphate which was present. The incubation mixture was chromatographed on a Dowex-1 column (formate) and the esters eluted with 0.1 M ammonium formate, pH 3. The pentose phosphate fraction was chromatographed on paper according to the procedure of Scott and Cohen ('51). Ribose-5-phosphate and xylose-5-phosphate moved with approximately the same R_f in ethanol-acetic acid (table 7). The migration of xylose-5-phosphate was not retarded by the boric acid, whereas ribose-5-phosphate and the isolated ester were both retarded. A small amount of material moves (with considerable smearing) ahead of the main spot and may represent uncomplexed ester. These data demonstrate that the

ester accumulating during xylose phosphorylation cannot be xylose-5-phosphate, but rather has the chromatographic properties of ribose-5-phosphate.

Preparations which phosphorylated xylose but did not degrade pentose phosphates were obtained either by suspending the cells in 0.05 *M* sodium bicarbonate during the sonic treatment or by treating the phosphate buffer extracts with manganese chloride to precipitate nucleic acids. When ribulose-5-phosphate was incubated with such extracts there was no change in total acid-soluble pentose, whereas with ribose-5-phosphate, a decrease of 10–20% was always observed. This suggests the presence of pentose isomerase in these extracts.

TABLE 7

Chromatography of isolated pentose phosphate

COMPOUND	R_f	
	Ethanol-Acetic acid	Ethanol-Boric acid
Ribose-5-phosphate	0.23	0.02 (0.31)
Xylose-5-phosphate	0.26	0.31
Pentose phosphate	0.24	0.0 (0.15)
Ribose-5-phosphate + pentose-phosphate	0.24	0.02 (0.1–0.2)

Figure 7 presents the data of a typical experiment with these preparations. ATP was added here in substrate amounts. It is obvious that approximately 2 moles of xylose were phosphorylated per mole of ATP disappearing. Whether ADP is active in xylose phosphorylation in this system or whether the extract contains a myokinase is not known at present.

The adenyl compounds were removed from the mixture by adsorption on IR-100 (H^+) resin in 0.1 *M* acetic acid and the pentose phosphate isolated from the eluate as a Ba^{++} salt. The preparation contained pentose, reducing sugar, and organic phosphorus in about 1 : 1 : 1 ratio. On treatment of the ester with bromine and barium carbonate for 15 minutes, 85% of the pentose-reacting material was destroyed. The remaining "pentose" was resistant to bromine and gave an

atypical color in the orcinol test for pentose. On treatment of the ester with sodium metaperiodate, about 2 moles of periodate were consumed per mole of ester within one minute at 25°C. and a value of 3 moles was obtained at 10 minutes and at 120 minutes. This is the value expected for a pentose-5-phosphate ester. During the periodate oxidation about 0.25 mole of formaldehyde formed per mole of ester. Oxidation of a 3-phosphate ester would result in the formation of 1 mole of formaldehyde per mole of ester. This indicated that the isolated ester consisted of pentose-5-phosphates with the small amount of formaldehyde originating from carbon-1 of keto sugars.

To obtain further data on the sugars present in the molecule, a sample of the ester was hydrolyzed with an acid phos-

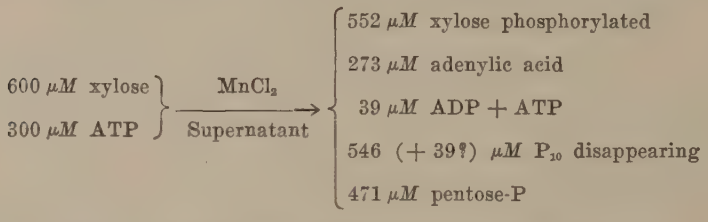

Fig. 7 Xylose phosphorylation in HCO_3 — sonic extract.

phatase from potato, the mixture deionized over ion-exchange columns, and the free sugars obtained in about 60% yield. This mixture of sugars has been chromatographed on paper in a number of solvent systems and on Dowex-1 (borate) columns. Since the more qualitative data obtained by chromatography on paper agreed well with the quantitative results from the columns, only the latter will be presented. Figure 8 presents the protocols of a typical experiment. Of the 39.6 μ*M* of pentose in the initial mixture, 31 μ*M* (78%) was present in the first, larger peak. This material has been identified as D-ribose by its position on the elution diagram and its properties on chromatography on paper in 5 solvent systems. The rest of the material eluted in a region intermediate between the positions of ribose and arabinose. In the orcinol

test for pentoses a spectrum was obtained with this substance which, in addition to the usual maxmium at 660–670 mμ, has a peak at 540–550 mμ. This has been shown by Scott and Cohen ('51) to be characteristic of keto pentoses. In paper chromatograms only a diffuse spot could be obtained with this fraction. Spraying the chromatograms with the heptulose reagent of Bevenue and Williams ('51) gave a blue color over a portion of the area covered by the fraction. It is probable

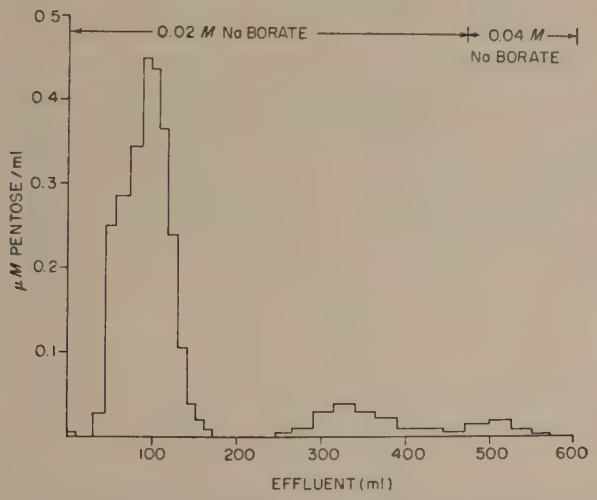

Fig. 8 Chromatography of pentose from isolated pentose phosphate. 39.6 μM of pentose in 0.02 M sodium borate absorbed on column of Dowex-1 (borate). Column size: 1.13 $cm^2 \times 8.5$ cm. R_f: ca. 0.5 ml/minute.

that this ketose fraction represents a mixture of ketopentose phosphates and heptulose phosphate in the initial pentose phosphate preparation.

It is clear that ribose phosphates can be formed by the action of the enzyme preparation on xylose and ATP. This is in agreement with the hypothesis that fermentation of D-xylose involves the intermediate formation of ribose derivatives. It should be emphasized that ribose-5-phosphate, although it was the ester which accumulated in these experiments where pentose removal was eliminated, may well not be the ribose es-

ter involved in xylose degradation. The results obtained with 1-C^{14}-D-xylose indicated a 2-ketopentose as an intermediate. If ribulose-5-phosphate were formed in the experiments with xylose and ATP, ribose-5-phosphate probably would have accumulated, since the enzyme preparations appear to contain the pentose phosphate isomerase and since Horecker et al. ('51) have shown that the equilibrium mixture contains 70–80% ribose-5-phosphate.

The intermediate stages in the conversion of xylose ($^+$ATP) to a ribose phosphate are at present obscure. Xylose-5-phosphate is not degraded by extracts of *L. pentosus* and hence is probably not an intermediate. Also we have been unable to obtain any evidence for the formation of acid-labile esters, such as xylose-1-phosphate (presumably) or ribose-1-phosphate; in fact, added ribose-1-phosphate is not degraded by these systems (Lampen, unpublished observations). This question is now under investigation.

DISCUSSION

HORECKER: In connection with the observations that Doctor Lampen has made, I think some results obtained by Seymour Cohen, about which he has written us, might be worth mentioning. From arabinose-adapted organisms, he is able to isolate an enzyme which converts arabinose to an equilibrium mixture of arabinose and ribulose. Apparently there is a conversion which precedes phosphorylation of the sugar, and it is possible that a similar situation exists in the phosphorylation of xylose.

LAMPEN: The only thing we tried was incubation of xylose with the sonic extracts. We obtained no significant alteration in the total amount of pentose, as one might expect if there were a significant amount of ketopentose accumulating, and no change in the pentose spectrum.

HORECKER: You do not know what the equilibrium is in that case?

LAMPEN: We have no idea what it is.

HORECKER: There may be only 20% of ketose, or less.

LAMPEN: Twenty per cent, with only 20–30% reduction in color value, would not be detected. The formation of a ketopentose is quite possible, I believe. It seems unlikely that ribose itself is an intermediate. Aged cells (grown on xylose) ferment xylose at several times the initial rate obtained with ribose, and also extracts of xylose-grown cells phosphorylate xylose several times as rapidly as ribose. A ketopentose, either xylulose or ribulose, could be the initial product in xylose fermentation, however.

KELLY: Are these pentose degradations reversible?

LAMPEN: As yet we have not studied the incorporation of any small molecules, such as in bacteria. In the animal there has been some work in Doctor Wood's laboratory with formate and acetate, the evidence there being that most of the pentose in the polynucleotides of the internal organs of the chick probably could not have arisen by a C_6 to C_5 pathway. However, it could have arisen at least in part by the C_2–C_3 condensation, but certainly did not necessarily do so.

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ROUTES OF ETHANOL FORMATION IN BACTERIA

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FOUR FIGURES

The history of bacterial fermentations is long and well described in terms of gross observations (Porter, '46). However, until very recently, except for a few familiar species, a detailed understanding of the intermediary metabolic systems involved has been almost nonexistent. Ethanol is a minor end product in numerous bacterial fermentations and, in a few instances, constitutes a major if not the sole nongaseous end product. It is thus apparent that a clear description of the bacterial mechanism for ethanol formation would be a valuable contribution to the definition of bacteria and to the study of comparative biochemistry. In view of the evidence for participation of the Embden-Meyerhof (E-M) system in ethanol formation by yeast, the existence of a similar mechanism in those bacterial species which produce ethanol seemed a logical question for consideration.

Although early data concerning glucose and pyruvate dissimilations by bacteria indicated that carboxylase, in addition to certain enzymes of the E-M system, participated in ethanol fermentations, the experimental methods available could not distinguish between possible alternative metabolic pathways. The introduction of more definitive enzymatic methods and the use of isotopic substrates has stimulated the reinvestigation and resolution of certain unsolved problems, e.g., the recent descriptions of the hexose monophosphate pathway in yeast (Horecker, '51) and *Escherichia coli* (Scott

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and Cohen, '51), and the discovery of previously unsuspected problems.

Our interest in the mechanism for ethanol formation by bacteria has led to experiments with *Leuconostoc mesenteroides* and *Pseudomonas lindneri,* taxonomically unrelated species which form ethanol in large quantities during glucose fermentation according to equation (1) of Gayon and Dubourg (1894, '01) and equation (2) of Kluyver and Hoppenbrouwers ('31).

L. mesenteroides

$$1.0\ \text{Glucose} \rightarrow 1.0\ \text{lactic acid} + 1.0\ \text{ethanol} + 1.0\ CO_2 \qquad (1)$$

P. lindneri

$$1.0\ \text{Glucose} \rightarrow 0.2\ \text{lactic acid} + 1.8\ \text{ethanol} + 1.0\ CO_2 \qquad (2)$$

The evidence obtained indicates the existence of anaerobic hexose monophosphate pathways which differ somewhat from the aerobic systems of yeast and *E. coli.*

LEUCONOSTOC MESENTEROIDES

Leuconostoc mesenteroides is a representative of the heterofermentative lactic acid group of bacteria which produce substantial quantities of ethanol and lactic acid from glucose. The various fermentations effected by species of this group have been analyzed by Gayon and Dubourg (1894, '01), Peterson and Fred ('20), Pederson ('29), Nelson and Werkman ('40), and others.

In table 1 are presented analyses of the products of glucose fermentations by *L. mesenteroides* at pH 7 and 4.5. The end products were formed in practically equimolar quantities in both experiments. Gunsalus and Niven ('42), using *Streptococcus faecalis,* reported a change in products of glucose fermentation dependent upon hydrogen ion concentration. At lower pH levels, a normal homolactic fermentation was produced, while at pH 9, formic and acetic acids were formed with a concomitant decrease in the yield of lactic acid. The constancy of *L. mesenteroides* fermentation products at differing pH levels suggested the participation of a reaction sequence different from the fermentative pathway of *S. faecalis.*

Although the hydrogen-ion concentration had no apparent effect upon the fermentation products, the rate of fermentation was altered considerably. This point will be discussed.

In order to elucidate the enzymatic makeup of the *L. mesenteroides* system, experiments were undertaken with cell-free extracts of the organism. Hexokinase, phosphoglyceric transphosphorylase, and dehydrogenases for D-glyceraldehyde phosphate, D-lactic acid, and ethanol were found to be active in extracts prepared by sonic disintegration. Utilizing the methods of Meyerhof and Lohmann ('34) for alkali-labile

TABLE 1

Glucose fermentation by Leuconostoc mesenteroides

	pH 7.0	pH 4.5
Substrate (μM)		
Glucose	9.35	17.5
Products (μM)		
Lactate	8.30	18.4
Ethanol	8.95	14.7
Carbon dioxide	9.70	18.6
Carbon recovery (%)	93.6	98.3
O/R balance	1.08	1.27

Protocol: 100 μM phosphate; 20 μM glucose, 3.3 mg fresh cells (dry weight); total volume, 4.0 ml. Warburg respirometer, 28°C., nitrogen atmosphere. Reactions stopped after 6 hours by addition of 600 μM sulfuric acid.

phosphate and of Sibley and Lehninger ('49) for dihydroxyacetone phosphate chromogen, neither aldolase nor isomerase could be demonstrated.

These results were apparently contradicted by the observation that hexose diphosphate (HDP) could serve as substrate for diphosphopyridinenucleotide (DPN) reduction, presumably by formation of triose phosphate, since aldolase would be expected to participate in such a reaction. It was apparent that a mechanism differing from the E-M system was involved and the actual nature of the pathway was suggested by the isotopic glucose fermentation analyses of Gunsalus

and Gibbs ('52). Their results are summarized in figure 1, which shows the carbon atom relationships of products to substrate. These relationships obviously could not obtain if the E-M system were operating, in which case the aldehyde carbon atom of glucose would appear in ethanol (Koshland and Westheimer, '50). The isotope data suggested the participation of an anaerobic hexose monophosphate pathway, perhaps similar to the aerobic systems described for yeast and *E. coli*. Subsequently, DPN reduction was observed with HDP as substrate in extracts which lacked not only aldolase but also triose phosphate dehydrogenase. Explanation of the phenomenon was revealed by demonstration of a glucose-6-phos-

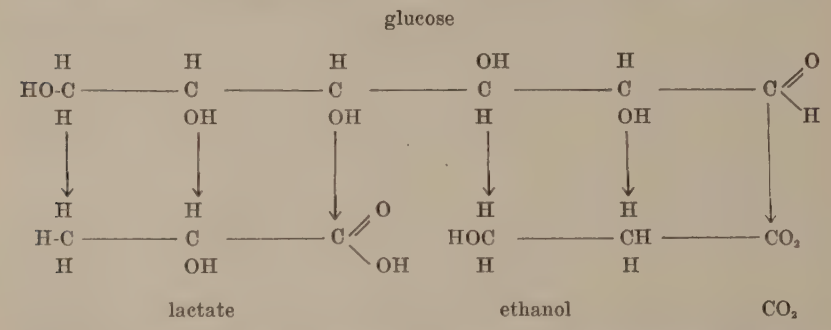

Fig. 1 *L. mesenteroides* fermentation: carbon atom relationships of products to substrate.

phate dehydrogenase. The enzyme was active with either DPN or TPN (triphosphopyridinenucleotide) as coenzyme, in contrast to the previously described yeast glucose-6-phosphate dehydrogenase which specifically requires TPN as coenzyme.

After 15-fold purification by ammonium sulfate fractionation and calcium phosphate gel treatment, some properties of the new glucose-6-phosphate dehydrogenase were determined. All active fractions obtained during the purification procedure catalyzed reduction of both DPN and TPN. Although other nonspecific pyridine nucleotide-linked dehydrogenases have been described (Black, '51; Mehler et al., '47), the possible occurrence of interfering reactions was tested. Transhydrogenation, the reversible transfer of electrons from TPNH

to DPN, has been observed in extracts of *Pseudomonas fluorescens* (Colowick et al., '52; Kaplan et al., '52). Kornberg ('50a, b) has described enzyme systems from yeast which catalyze the synthesis of DPN and TPN from the constituent components. The activity of either mechanism in *L. mesen-*

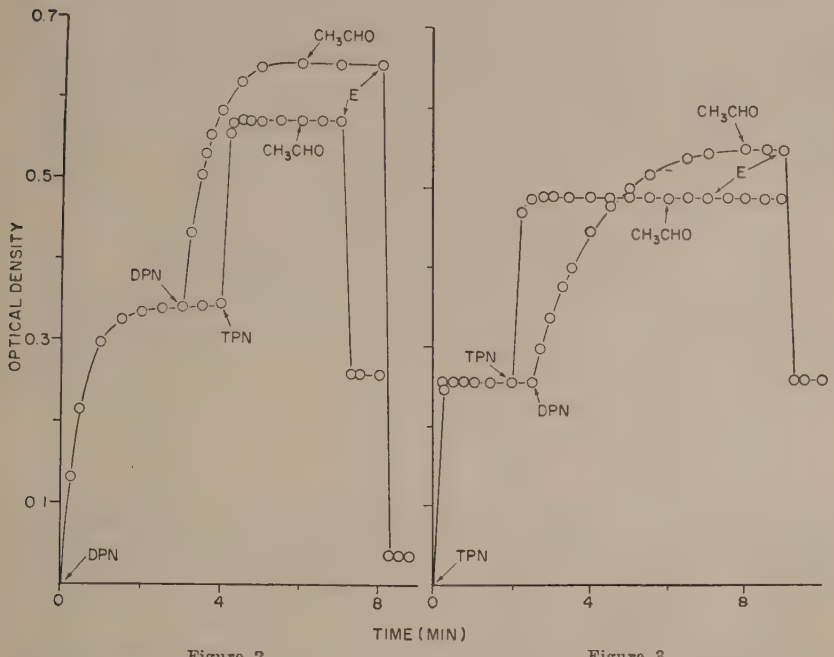

Figure 2 Figure 3

Fig. 2 (left) and Fig. 3 (right) *L. mesenteroides:* pyridine nucleotide specificity of glucose-6-phosphate dehydrogenase. Protocol: 1.5 ml veronal buffer, pH 7.8; 4 μM glucose-6-phosphate; glucose-6-phosphate dehydrogenase, 5 μg total protein; total volume 3 ml. 0.16 μM DPN; 0.14 μM TPN; 10 μM acetaldehyde and ethanol dehydrogenase (E) were added as indicated. Beckmann spectrophotometer, 340 mμ, 23°C.

teroides extracts would lead to the illusion of nonspecific coenzyme requirement for the glucose-6-phosphate dehydrogenase. The results presented in figures 2 and 3 show that neither of these interfering mechanisms are effective under the experimental conditions employed. In these experiments, after complete reduction of the pyridine nucleotides, addition of

acetaldehyde and crystalline yeast ethanol dehydrogenase resulted in oxidation of reduced DPN (DPNH) only. The extent of DPNH oxidation in each experiment corresponded closely to the quantity of DPN originally added, showing that no nucleotide interconversion or transhydrogenation had occurred. The nonspecific coenzyme requirement was further substantiated by the constant ratio (0.67) of rate of reduction with DPN to that with TPN obtained for all fractions during the purification procedure.

Aqueous solutions of the glucose-6-phosphate dehydrogenase are stable for at least three days at 25°C. and for at least 8

TABLE 2

Effect of phosphate on glucose fermentation rate
Leuconostoc mesenteroides

PHOSPHATE MOLARITY	Q_{CO_2}	INHIBITION
		%
0.0	212	
9.0×10^{-4}	49	77
4.5×10^{-3}	32	85
9.0×10^{-3}	26	88

Protocol: 2.0 ml veronal buffer, pH 7.0; 5.9 mg fresh cells (dry weight); 10 μM glucose, phosphate, pH 7.0, as indicated; total volume, 3.7 ml. Warburg respirometer, 28°C., nitrogen atmosphere.

months at — 20°C. The TPN-specific yeast glucose-6-phosphate dehydrogenase is relatively unstable at 0°C. (Negelein and Gerischer, '36; LePage and Mueller, '49). The maximum rate of pyridine nucelotide reduction was obtained at pH 7.8 in veronal buffer. Optimum pH for activity of yeast enzyme has not been reported although Negelein and Haas ('35) employed phosphate buffer of about pH 7.5 in their studies. The *Leuconostoc* enzyme is similar to yeast glucose-6-phosphate dehydrogenase in inhibition by phosphate (Theorell, '34) and in the stimulatory effect of magnesium ions (Kornberg, '50b).

The observed phosphate inhibition may partially explain fermentation data obtained with cell suspensions (DeMoss, Bard, and Gunsalus, '51). Addition of orthophosphate to re-

action mixtures in veronal buffer at pH 7 caused a marked inhibition of the fermentation rate (table 2). In this connection the rate of fermentation in phosphate buffer was also dependent upon hydrogen-ion concentration. Table 3 shows that a 27-fold increase in the rate of carbon dioxide evolution (corrected for carbon dioxide retention) was obtained by decreasing the pH from 7 to 5. The activity of the fermentative mechanism of *L. mesenteroides* thus appears to be favored by relatively high hydrogen-ion concentrations. A similar pH effect has been observed during *E. coli* glucose fermentations

TABLE 3

Effect of pH on glucose fermentation by Leuconostoc mesenteroides and Escherichia coli

pH	GLUCOSE Q_{CO_2}	GLUCOSE-1-C^{14} TOTAL ACTIVITY OF C^{14} in CO_2
		%
4.5	...	15
5.0	134	..
5.5	...	0
6.5	...	0
7.0	5	..

Protocols: *L. mesenteroides:* 3.7 mg fresh cells (dry weight); 66 μM phosphate; 10 μM glucose; total volume, 3.0 ml. Warburg respirometer, 30°C., nitrogen atmosphere. *E. coli:* 5.0 mg fresh cells (dry weight); 17 μM phosphate; 10 μM glucose-1-C^{14} (11 mμc C^{14}); total volume, 3.0 ml. Warburg respirometer, 30°C., nitrogen atmosphere.

by Gest and Gibbs ('51, unpublished data). In *E. coli,* the hexose monophosphate pathway apparently participates in anaerobic glucose dissimilation under certain conditions. During glucose-1-C^{14} fermentation in phosphate buffer at pH 4.5, the evolved carbon dioxide contained 15% of the total isotopic carbon while at pH 5.5 or 6.5 no C^{14} appeared as carbon dioxide (table 3).

In the hexose monophosphate pathways of yeast and *E. coli,* 6-phosphogluconate is oxidized and decarboxylated to form a pentose phosphate. However, results of the following experiments with *L. mesenteroides* extracts, in conjunction with the

isotope data, indicate that pentose phosphate is not an intermediate in 6-phosphogluconate dissimilation, but that the actual mechanism may involve a cleavage of a keto-6-phosphogluconic acid. With added yeast hexokinase, crude enzyme extracts catalyzed carbon dioxide evolution from glucose; pentose phosphate did not accumulate. However, with the same extracts, neither ribose-5-phosphate nor ribulose-5-phosphate (kindly furnished by Dr. B. L. Horecker) were consumed. Under the same experimental conditions, similar extracts of *E. coli* did catalyze the disappearance of ribose-5-phosphate.

One hypothetical mechanism, consistent with the data but not involving pentose phosphate, would include oxidation, cleavage, and decarboxylation reactions starting with 6-phosphogluconate. Formation of triose phosphate as a product of the cleavage reaction is a reasonable assumption in view of (a) the compatibility of the isotopic glucose fermentation data with such a reaction, and (b) the occurrence of triose phosphate as a cleavage product in hexose and pentose metabolism (Meyerhof and Lohmann, '34; Racker, '48). If oxidation occurs first, triose phosphate and hydroxypyruvate might be expected to be products of the cleavage reaction. On the other hand, if cleavage of phosphogluconate is the primary reaction, triose phosphate and glyceric acid could occur as intermediate compounds. The following experiments represent preliminary attempts to test the above hypotheses and to elucidate the actual mechanism involved.

Certain crude extracts catalyzed the reduction of either DPN or TPN with phosphogluconate as substrate. Ammonium sulfate fractionation resulted in a 20-fold purification of the enzymatic activity and DPN was specifically required as coenzyme for the purified system. Triose phosphate dehydrogenase activity was absent from these preparations and glyceric acid was inactive as a substrate for DPN reduction. When tested manometrically with added acetaldehyde and ethanol dehydrogenase, the rate of carbon dioxide evolution (0.78 μM/hour/mg P) catalyzed by the purified preparations was negligible in comparison to the rate of DPN reduction (6.9 μM/

hour/mg P) observed spectrophotometrically (table 4), indicating that the oxidative reaction precedes decarboxylation.

The following experiments also suggest that oxidation precedes cleavage of 6-phosphogluconate. If the purified 6-phosphogluconate dehydrogenase preparation catalyzes triose phosphate formation either before or after 6-phosphogluconate oxidation, the reaction should be detectable as carbon dioxide evolution in bicarbonate buffer after conversion of the triose phosphate to phosphoglycerate. Components of the reaction system included bicarbonate buffer, 6-phosphogluconate as substrate, purified 6-phosphogluconate dehydrogenase to cata-

TABLE 4

Rates of carbon dioxide evolution and DPN reduction with 6-phosphogluconate by Leuconostoc mesenteroides extracts
(μM/hr./mg P)

DPN	CO_2
6.93	0.78

Protocols: CO_2 evolution: 100 μM phosphate; 0.39 μM DPN; 12 μM 6-phosphogluconate; crude extract (4.8 mg protein); 0.2 μM cocarboxylase; 250 μM acetaldehyde; ethanol dehydrogenase (50 μg protein); total volume, 3.0 ml; pH 7.0. Warburg respirometer, 30°C., nitrogen atmosphere.

DPN reduction: 150 μM phosphate; 0.59 μM DPN, 4.0 μM 6-phosphogluconate; crude extract (0.96 mg protein); 0.2 μM cocarboxylase; total volume, 3.0 ml; pH 7.0. Beckman spectrophotometer, 340 mμ, 24°C.

lyze oxidation and possibly cleavage of the substrate, triose phosphate dehydrogenase to catalyze oxidation of triose phosphate if formed, DPN as electron carrier, acetaldehyde and ethanol dehydrogenase for oxidation of DPNH and magnesium ions, arsenate, and cysteine as activators for the system. Each enzyme component was active when tested alone under similar conditions. With the complete system, no acid was formed, indicating that either triose phosphate is not a cleavage product or, more probably, the enzyme which catalyzes the cleavage reaction is not present in the preparation used. Thus the reaction sequence, starting with 6-phosphogluconate, is visualized as oxidation, cleavage, and decarboxylation.

Other hypothetical mechanisms which would involve pyruvate as an intermediate appear unlikely in the light of the isotope data. The identity of glucose carbon atom 3 with the carbinol carbon of ethanol precludes a simple decarboxylation of pyruvate. Lack of mixing of glucose carbon atom 1 with the carboxyl carbon of lactate also argues against the occurrence of pyruvate as an intermediate.

PSEUDOMONAS LINDNERI

Pseudomonas lindneri is a gram-negative, short, rod-shaped bacterium, isolated from Mexican pulque, an alcoholic beverage. An atypical member of the genus, other members of which exhibit strictly oxidative properties, *P. lindneri* produces an essentially yeast-type fermentation of glucose. The fermentative activities of this organism have been reported by Kluyver and Hoppenbrouwers ('31), Kluyver ('31), Neuberg and Kobel ('31), and Tankó ('32).

The experimental results to be reported here indicate that an anaerobic hexose monophosphate pathway participates in glucose fermentation by *P. lindneri* with the added feature of a carboxylase reaction. A preliminary report has appeared (Gibbs and DeMoss, '51). Analysis of isotopic glucose and fructose fermentations gave results which are presented in tables 5 and 6. Glucose carbon atoms 1 and 4 yield carbon dioxide, while ethanol is derived from the remainder of the glucose molecule. The appearance of glucose carbon atom 3 in both carbon atoms of ethanol indicates the participation of a mechanism different from that operating in *L. mesenteroides*. The nature of the difference in mechanisms is not yet apparent, although the following two possibilities can probably be eliminated. The data cannot be explained simply on the basis of occurrence of a symmetrical 2-carbon intermediate, since maximum participation of such a compound would result in equal distribution of the isotope activity between the two ethanol carbon atoms. Participation of pyruvate and carboxylase in the metabolism of C_1, C_2, and C_3 of glucose would be compatible with the identity of C_3 in the

methyl carbon atom of ethanol, but could not alone explain the appearance of C_3 in the carbinol carbon atom of ethanol.

The apparent identity of fructose carbon atom 6 with the methyl carbon of ethanol agrees with the reported existence of carboxylase in *P. lindneri* (Neuberg and Kobel, '31). Car-

TABLE 5

Isotopic sugar fermentation in Pseudomonas lindneri

SUBSTRATE	C^{14} (mμc)		
	SUBSTRATE	PRODUCTS	
		CO_2	Ethanol
Glucose-1-C^{14}	15	16	0.05
Glucose-1-C^{14}	15	15.1	0.09
Glucose-3,4-C^{14}	14.8	7.5	7.5
Fructose-1,6-C^{14}	..	30.5	22.3
Fructose-1,6-C^{14}	..	28.6	25.2

Protocols: 100 μM phosphate; 5 mg fresh cells (dry weight); 10 μM substrate; total volume, 2.1 ml; pH 4.6. Warburg respirometers, 30°C., nitrogen atmosphere. After complete reaction, CO_2 and ethanol collected and combusted (Gunsalus and Gibbs, '52).

TABLE 6

Position of C^{14} in ethanol (Pseudomonas lindneri)

SUBSTRATE	ETHANOLIC C^{14} (%)	
	CH_3	CH_2OH
Glucose-3,4-C^{14}	89	11
Glucose-3,4-C^{14}	88	12
Fructose-1,6-C^{14}	95	5

Protocols: same as table 5. Ethanol oxidized by yeast ethanol dehydrogenase and acetaldehyde trapped as bisulfite addition product. Acetaldehyde released by distillation from dibasic potassium phosphate solution and degraded with alkaline iodine.

boxylase was partially purified from crude extracts and resolved by precipitation with ammoniacal ammonium sulfate according to the method of Green, Herbert, and Subrahmanyan ('41). The acetoin-forming system, reported previously by Tankó ('32), which interfered in the manometric assay for carboxylase, was either removed or inactivated by the alka-

line precipitation. Table 7 shows that resolution of carboxylase was essentially complete.

Glucose fermentation by crude extracts of *P. lindneri* with added yeast hexokinase yielded approximately 1.5 moles of carbon dioxide per mole of glucose used, indicating that the complete fermentative mechanism was present. Pentose phosphate did not accumulate, and was not metabolized — results similar to those obtained with *L. mesenteroides*. Uniformly labeled glucose was fermented by *P. lindneri* extracts, and the reaction mixture chromatogrammed after deproteinization and enzymatic hydrolysis of the phosphate esters. The phos-

TABLE 7

Resolution of carboxylase activity (P. lindneri)

ADDITIONS	RATE OF CO_2 PRODUCTION
	μl/30 min.
None	15
Mg^{++}, 1.6×10^{-3} *M*	11
Cocarboxylase, 3.3×10^{-4} *M*	23
Mg^{++} + cocarboxylase	361

Protocol: 150 *μM* citrate; 500 *μM* pyruvate; 0.2 ml purified enzyme preparation; total volume, 3.0 ml; pH 6.0. 5.0 *μM* magnesium sulfate and 1 *μM* cocarboxylase added as indicated. Warburg respirometer, 30°C., nitrogen atmosphere.

phatase preparation (Polidase) was added to the reaction mixture after inactivation of the latter at 100°C. and adjustment to pH 5.5. Figure 4 represents graphically the results of exposure of the chromatogram to x-ray film and the aniline hydrogen phthalate spray test which determines pentoses and hexoses.

The spot representing compound 1 contained no isotopic carbon and shows the presence of glucose in the phosphatase preparation used. The glucose spot is not present in chromatograms of unhydrolyzed reaction mixtures. Compound 2 possesses R_f values similar to those of fructose and probably represents unfermented fructose which was present as an impurity in the isotopic glucose substrate solutions.

Compounds 3 and 4 gave positive pentose reactions and contained no isotopic carbon, indicating that they were not intermediates in the reaction sequence but were formed during hydrolysis of other pentose-containing compounds.

Compound 5 contained isotopic carbon and is therefore a suspected intermediate product. Some properties of the substance may be deduced from its behavior on chromatograms.

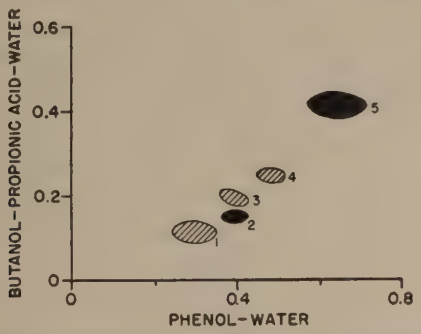

Fig. 4 *P. lindneri:* chromatogram of glucose-C^{14} dissimilation products. Protocol: 10 μM phosphate; 0.5 ml *P. lindneri* crude extract; 50 μg yeast hexokinase; 13.9 μM glucose-C^{14} (1.3 μc C^{14}); total volume, 1.3 ml; pH 4.6. Warburg respirometer, 30°C., nitrogen atmosphere. ▤ contains C^{14}. ■ aniline-H-phthalate spray. Cup contents heated three minutes at 100°C. after 23.8 μM carbon dioxide evolved. 50 mg Polidase added to 0.5 ml inactivated reaction mixture, incubated 6 hours at 37°C. 1 ml 10% trichloroacetic acid added, solution dried *in vacuo* over calcium chloride. Residue washed three times with 0.3-ml portions of water, resulting solution dried *in vacuo* over calcium chloride. Residue dissolved in 0.2 ml water, 13 μl spotted on 36 × 57 cm Whatman no. 1 paper, irrigated with phenol (72% vol/vol)-water, then with butanol-propionic acid-water (62.5: 31: 43.5 by vol). Compounds located by exposure to Type K industrial x-ray film (Eastman Kodak) or by the aniline-hydrogen-phthalate spray test (Partridge, '49).

It is relatively nonvolatile and is probably nonacidic. Acidic substances in general possess low R_f values in the aqueous phenol solvent used. The compound is not a phosphate ester since the same relative position obtained whether the reaction mixture was chromatogrammed before or after phosphatase treatment. Compound 5 was not identifiable with mannitol, a possible product of fructose reduction, or with acetoin, a possible fermentation end product, or with glycolaldehyde,

a possible intermediate. The R_f values are similar to those of ethylene glycol and sedoheptulose. The occurrence of sedoheptulose is unlikely in view of the inactivity of the *P. lindneri* extract toward pentose phosphates. Other possible intermediate substances have not been tested for identity with the unknown compound.

The experiments reported, while not providing final description, do indicate the general outlines of the anaerobic hexose monophosphate pathways which participate in ethanol formation by *L. mesenteroides* and *P. lindneri*. The critical experiments needed for elucidation of the mechanisms are implied, although the question of the 2-carbon intermediates and the reactions leading to ethanol formation may provide considerable difficulty. The isotopic data indicate that the pathways followed by C_1, C_2, and C_3 of glucose may be somewhat different in the two organisms.

Participation of acetate as an intermediate is uncertain, although free acetate not only stimulates the rates of glucose fermentation (DeMoss, Bard, and Gunsalus, '51) but enters the fermenting system of *L. mesenteroides* (Gunsalus and Gibbs, '52). *L. mesenteroides* possesses an enzyme system which catalyzes acetyl phosphate formation from acetate and adenosinetriphosphate (DeMoss and Gunsalus, '49). This mechanism could obviously facilitate the entry of added acetate into the 2-carbon pathway but would probably not participate in the internal system.

ACKNOWLEDGMENT

The author gratefully acknowledges the encouragement and help of Dr. Martin Gibbs, who performed many of the isotope experiments reported.

DISCUSSION

H. G. WOOD: I should like to ask a question about the spot no. 5 that contained carbon-14 (fig. 4). You say that you do not think it could be sedoheptulose because pentose phosphate is not metabolized.

DeMoss: That is correct, and, in addition, a positive test was not obtained with the orcinol spray reagent.

Carson: Doctor DeMoss, did you or Doctor Gibbs ever ferment 3,4-labeled glucose with *Leuconostoc,* and if so, have you observed differences in the specific activities of the carbinol group of ethanol and the lactate carboxyl (these carbons corresponded to the 3,4 carbons of the sugar)?

Gibbs: We have noticed a difference between the specific activities of lactic acid and ethanol arising from fermentations of glucose-3,4-C^{14} with *L. mesenteroides,* but we have attributed this difference to the difficulty in isolating the small amounts of ethanol. These experiments are carried out by collecting the carbon dioxide in the side arm of the Warburg cup, centrifuging out the organism, and then removing the alcohol by distillation. During these operations traces of alcohol are easily lost.

However, in *P. lindneri,* only carbon dioxide and ethanol are obtained. The carbon dioxide is collected in the side arm and the alcohol is oxidized immediately. The specific activities are about the same.

The glucose-3,4-C^{14} used in these experiments was synthesized by injecting a rat with radioactive bicarbonate, isolating the glucose from the rat liver glycogen. When this glucose is chemically degraded, all the activity is located in carbon atoms 3 and 4.

However, when glucose is used in microbial fermentations, small amounts (about 3%) do not appear in the expected positions of the fermentation products. This occurs with *Lactobacillus casei* and *Pseudomonas lindneri.* This is not due to errors in the degradations.

This would indicate that the mechanism in the rat whereby C^{14} is placed into carbon atoms 3 and 4 of glucose is somewhat different from the mechanism whereby the microorganism degrades it.

Noggle: We have had some experience with 3,4-labeled glucose that may be of interest here. We used the preparative procedure of Ward, Lifson, and Lorber and, following acid

hydrolysis of the rat liver glycogen, passed the glucose through cation- and anion-exchange columns to remove impurities. When the 3,4-glucose was degraded with the *Leuconostoc* fermentation scheme of DeMoss, Bard, and Gunsalus, we found the sample to contain extra carbon that was not fermented by the *Leuconostoc*. The glucose sample was passed through a charcoal column to remove disaccharide and polysaccharide compounds. Roughly, one-half the radioactivity of the original sample remained on the column. The glucose when then degraded gave essentially 100% recovery of the radioactivity. The activity in the charcoal column was eluted and a paper chromatogram made of this fraction showed a spot with an R_f lower than sucrose. Chemical tests of the material indicated a polyglucose compound.

DEMOSS: *L. mesenteroides* is employed for commercial production of dextran. If any sucrose were present in your preparation, polysacharide formation might be expected. However, dextran is not reported to be formed by *L. mesenteroides* from either glucose or fructose.

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EVIDENCE FROM FERMENTATION OF LABELED SUBSTRATES WHICH IS INCONSISTENT WITH PRESENT CONCEPTS OF THE PROPIONIC ACID FERMENTATION [1]

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The propionic acid bacteria ferment a number of substrates and the products in each instance are propionate, acetate, succinate, and carbon dioxide. Perhaps the most striking feature of the propionic acid fermentation is that these same end products are obtained from the fermentation of 3-, 4-, 5-, and 6-carbon sugars and polyalcohols. It is mainly the ratios of these end products which vary from one substrate to another, and this variation seems to be more dependent upon the degree of oxidation or reduction of the substrate than upon the number of carbons in the substrate. Up to the present time the mechanism of fermentation of the 4- or 5-carbon sugars or polyalcohols has not been studied extensively.

There is considerable evidence in the literature that the Meyerhof scheme is in operation in the fermentation. This evidence will not be given here; only those observations which indicated that an additional mechanism of fermentation occurs will be mentioned.

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It is well known that the phosphopyruvate enolase enzyme is very sensitive to fluoride, and use of this fact was made to determine if phosphoglyceric acid (PGA) was an essential intermediate. Werkman, Stone, and Wood ('37) grew the propionic acid bacteria in the presence of fluoride, and it was shown that the products were the same as in the absence of fluoride. Under the same conditions in the presence of fluoride, the bacteria did not ferment PGA (table 1). The medium contained 2% glucose and 1% phosphoglycerate (PGA). The days of growth are 0, 4, and 14 and the values are milligrams per milliliter. It is seen that at 0 time there were about 22 mg per ml of glucose present and that in either the presence or absence of fluoride the glucose was rapidly utilized, being

TABLE 1

DAYS	NO NaF		NaF ADDED (0.02 *M*)	
	Glucose	Inorganic P	Glucose	Inorganic P
	mg	*mg*	*mg*	*mg*
0	21.8	0.16	22.4	0.16
4	8.6	0.42	11.5	0.12
14	0.5	1.83	0.3	0.21
	Total phosphate	1.89		1.88

almost gone in 14 days. The utilization of PGA in the same experiment was followed by determining the inorganic phosphate liberated from the phosphate ester. It is seen that, in the absence of fluoride, there was liberation of phosphorus and about all the total phosphorus of the medium had been converted to inorganic phosphate in 14 days whereas, in the presence of fluoride, the phosphorus remained largely in esterified form and the PGA was not utilized.

This experiment therefore showed that PGA was utilized, but it also showed that fluoride stops the utilization and that fluoride does get into the cell. However, glucose utilization occurred whether or not PGA utilization was stopped. Thus the results indicated that fluoride is effective and that glucose can be utilized by a pathway that does not involve PGA. So

it seemed quite clear that glucose could be metabolized by two mechanisms, only one of which involved PGA.

Our first studies concerned the role of carbon dioxide fixation and whether or not propionate is formed by decarboxylation of succinate. The question of whether or not propionate was formed by direct reduction or by a decarboxylation of a symmetrical C_4 dicarboxylic acid has remained unsettled even though support of the latter has been increasing. If the decarboxylation of a symmetrical C_4 dicarboxylic acid were involved there would be 1 mM of carbon dioxide fixed and 1 mM evolved for every mM of propionate formed as shown in equations (1a) and (1b). Since there is an equal

$$CH_3\text{–}\overset{\overset{O}{\|}}{C}\text{–}COOH \xrightarrow{C^*O_2} HOOC^*\text{–}CH_2\text{–}CH_2\text{–}COOH \qquad (1a)$$

$$HOOC^*\text{–}CH_2\text{–}CH_2\text{–}COOH \begin{cases} \nearrow C^*O_2 + CH_3\text{–}CH_2\text{–}COOH \\ \searrow HOOC^*\text{–}CH_2\text{–}CH_3 + CO_2 \end{cases} \qquad (1b)$$

chance of either end of the succinate being decarboxylated, the $C^{14}O_2$ or $C^{12}O_2$ turnover would be one-half the total carbon dioxide turnover.

We studied the carbon dioxide turnover by conducting the fermentation in the presence of a pool of labeled $C^{14}O_2$ and determining the dilution of C^{14} in the pool and the activity of the propionate. From these activities it was possible to calculate the $C^{14}O_2$ fixed per 100 mM of propionate produced.

TABLE 2

SUBSTRATE	mM $C^{14}O_2$ FIXED PER 100 mM PROPIONATE FORMED
Glycerol	12
Pyruvate	6
Erythritol	14
Adonitol	21

It can be seen (table 2) that the amount of $C^{14}O_2$ fixed is much less than 50 mM per 100 mM of propionate produced. It did not seem likely that the observed low amount of carbon

dioxide fixed was due to cell impermeability or to an incomplete mixing of the carbon dioxide pool with the biologically formed carbon dioxide, although this could not be ruled out. It appeared more likely that some other mechanism was operating which did not require carbon dioxide.

It thus became of interest to determine whether or not propionate might not be formed by direct reduction rather than by decarboxylation of a symmetrical C_4 dicarboxylic acid. If such were the case one would expect that α-labeled pyruvate or lactate would give α-labeled propionate and β-labeled substrates would give β-labeled propionate; whereas, if propionate were formed by decarboxylation of succinate, the isotope would be randomized in the α and β positions. We therefore fermented pyruvate labeled in the carbonyl carbon and lactate labeled in the methyl carbon. The results given in equations (2a), (2b), and (2c) were obtained

$$\overset{100}{CH_3}\text{–}\overset{}{CO}\text{–}\overset{}{COOH} \longrightarrow \overset{39}{CH_3}\text{–}\overset{49}{CH_2}\text{–}\overset{13}{COOH} \qquad (2a)$$

$$\overset{100}{CH_3}\text{–}\overset{}{CHOH}\text{–}\overset{}{COOH} \longrightarrow \overset{43}{CH_3}\text{–}\overset{48}{CH_2}\text{–}\overset{8.5}{COOH} \qquad (2b)$$

$$\overset{100}{CH_3}\text{–}\overset{}{CHOH}\text{–}\overset{}{COOH} \longrightarrow \overset{41}{CH_3}\text{–}\overset{50}{CH_2}\text{–}\overset{9.2}{COOH} \qquad (2c)$$

with resting cells of *Propionibacterium arabinosum*. The values given above the carbons represent the specific activity relative to the activity of the labeled position of the substrate which has been assigned a value of 100. It can be seen that the α carbon had a higher specific activity than the β carbon even when methyl-labeled lactate was fermented, so that there was no indication of a direct reduction. The greater specific activity of the α carbon as compared to the β carbon of the propionate does not seem to have been due to an error in isolation or degradation. It is to be noted that the value of the α carbon is very close to 50, as is the sum of the β and carboxyl carbons. The value of 50 is to be expected if the propionate arises by decarboxylation of a symmetrical C_4 dicarboxylic acid. The fact that the sum of the activities of the β and carboxyl carbons is nearly 50 suggests that activity

may have been randomized from the β to the carboxyl carbon by equilibration of the propionate or a precursor with a symmetrical C_3 compound. By way of illustration, the mechanism shown in equations (3a), (3b), and (3c) would account for

$$C_1 + \overset{100}{C} - C - COOH \leftrightarrows COOH - \overset{100}{C} - C - COOH \qquad (3a)$$

$$COOH - \overset{100}{C} - C - COOH \leftrightarrows \overset{50}{C} - \overset{50}{C} - COX \leftrightarrows \overset{50}{CH_3} - \overset{50}{CH_2} - COOH \qquad (3b)$$

$$\overset{50}{C} - \overset{50}{C} - COX \leftrightarrows XOC - \overset{50}{C} - \overset{50}{C} - COX \leftrightarrows \overset{25}{CH_3} - \overset{50}{CH_2} - \overset{25}{COOH} \qquad (3c)$$

the observed distribution of C^{14}. It is seen that the randomization in the symmetrical C_3 compound would not alter the specific activity of the center carbon but would lower that of the β carbon, the extent of which would be determined by the proportion of the propionate formed via the symmetrical C_3. The fact that the α carbon of propionate had very nearly the required value of 50 [equations (2a), (2c)] makes it appear likely that propionate is formed via a C_4 dicarboxylic acid. However, if a C_4 dicarboxylic acid is indeed an intermediate in the formation of propionate, it seems necessary in view of our turnover studies, to assume that a C_1 other than carbon dioxide is involved in the decarboxylation. Presumably, the C_1 might be in partial equilibrium with carbon dioxide, and carbon dioxide fixation would occur via conversion to the C_1. This would account for the fact that the carbon dioxide turnover is lower than expected, since the C_1 could be used repeatedly.

It was decided to test the possibility that a 1-carbon intermediate, such as formic acid or formaldehyde, might occur in this reaction. In testing this possibility, we were much surprised to find that C^{14} of formaldehyde was readily incorporated into every position of all the end products isolated. Typical results are given in figure 1. These activities are arbitrarily presented on the basis of 100 counts in the

$$\begin{array}{cccc} & \overset{53}{CH_3} & - \overset{50}{CH_2} & - \overset{92}{COOH} \\ \overset{70}{HOOC} & - \overset{100}{CH_2} & - \overset{100}{CH_2} & - \overset{70}{COOH} \end{array}$$

Figure 1

ethylene carbon of succinate. This experiment represents a resting-cell fermentation of glycerol in the presence of a small amount of radioactive formaldehyde. It is to be noted that the ethylene carbons of the succinate contain approximately twice the activity of the ethylene carbons of the propionate. The average specific activity of the succinate is higher than that of the propionate, indicating that formaldehyde carbon in these experiments is incorporated to a greater degree in the succinate than in the propionate.

In order to show that formaldehyde was an intermediate, it was necessary to show that it was produced as well as utilized in at least one fermentation. We attempted to show its production by fermenting glycerol-1-C^{14} in the presence of an unlabeled formaldehyde pool [equation (4)].

$$\overset{100}{CH_2OH}\text{–}\overset{100}{CHOH}\text{–}\overset{}{CH_2OH} \longrightarrow \overset{100}{HCHO} + \overset{0}{HCHO} \longrightarrow \text{End products} \qquad (4)$$

Fermentation was stopped after a short time and before all the formaldehyde was utilized; the formaldehyde was reisolated and the activity determined. The reisolated formaldehyde had 80% of the specific activity of the terminal carbons of the glycerol, indicating that an appreciable amount of formaldehyde was produced. It would appear that formaldehyde has a reasonable turnover number and is probably an intermediate and that, in glycerol fermentations, it arises mainly, if not entirely, from the terminal carbon of the glycerol. It has been shown by Wood and Werkman ('37) that formaldehyde can be fixed from glucose fermentation when dimedon is used as a fixative.[3]

[3] The original report included data on the yield of formyl dimedon from total, 1-C^{14}-, and 3,4-C^{14}-glucose. Subsequent investigation has shown that the total labeled glucose probably was not pure and that part of the C^{14}-formyl dimedon in these fermentations may have arisen from impurities. The quantitative significance of the previous data, therefore, is questionable, and for that reason these data have been omitted from the present report. Further investigations are underway with sugars which have been further purified in an attempt to obtain data which are of quantitative significance.

It was previously concluded that a C_1 other than carbon dioxide might be involved in the formation of propionate from succinate and that carbon dioxide may be fixed by conversion to the C_1. The results on formaldehyde indicate that it too may be an intermediate, but formaldehyde enters every position and the carbon dioxide enters only the carboxyl groups. It therefore becomes necessary to postulate the role of two different C_1 compounds in this fermentation. It is known from recent studies of animal metabolism that carbon dioxide and formate or formaldehyde follow different pathways (Wood, '52).

The results obtained from the studies of formaldehyde metabolism naturally aroused our curiosity as to the distribution of labeling in the end products from the fermentation of labeled glucose, since it appeared that the fermentation might not yield products labeled according to predictions made from traditional schemes.

Up to this point two types of fermentation cleavages had received attention for glucose metabolism by bacteria, yeast, and animals: (a) the typical glycolytic cleavage to triose phosphate and then to phosphoglycerate and (b) the hexose monophosphate shunt via a C_1 cleavage to a pentose which in turn is converted to a triose. In the propionic acid fermentation of glucose-3,4-C^{14}, the labeling of the propionate and succinate would be expected to be entirely confined to the carboxyl carbons if either of these cleavages occurs. An example of the latter cleavage is very likely illustrated by the results of DeMoss and co-workers with *Leuconostoc mesenteroides* (This Supplement), by Lampen et al. ('51), and Rappoport and co-workers ('51) with *Lactobacillus* [equation (5)]. With *L. mesenteroides* the carbon dioxide arises

$$\begin{array}{l} \overset{0}{\underset{1}{C}} \vdots \text{——} \underset{2}{C} \text{——} \underset{3}{C^*} \vdots \text{——} \underset{4}{C^*} \text{——} \underset{5}{C} \text{——} \underset{6}{C} \\ \qquad\qquad\qquad\qquad\qquad\qquad \downarrow \\ \overset{0}{\underset{1}{CO_2}} + \underset{2}{C} \text{——} \underset{3}{C^*OOH} + \underset{4}{HOOC^*} \text{——} \underset{5}{C} \text{——} \underset{6}{C} \end{array} \qquad (5)$$

from the number 1 carbon, and the 3- and 4-carbons become

the carbinol carbon of ethyl alcohol and the carboxyl of lactic acid, respectively. If, in the propionate fermentation, the end products are modified so that the ethyl alcohol becomes acetic acid, as Lampen et al. found upon fermenting xylose-1-C^{14} with *L. pentosus,* then we would expect that the acetic acid would be labeled in the carboxyl group. The point to be noted is that, by either scheme — the usual glycolytic cleavage or the hexose monophosphate shunt — only carboxyl-labeled acids are obtained from 3,4-labeled sugars. By either type of split the succinate formed either by carbon dioxide fixation or by the Krebs cycle would be carboxyl labeled. From glucose-1-C^{14} one would expect little or no labeling in the carbon dioxide if the fermentation was via the glycolytic scheme, but with a mechanism such as given by *L. mesenteroides* the carbon dioxide would be active.

It therefore was of interest to study the fermentation of these two types of sugars. The results for glucose-3,4-C^{14} are given in figure 2. The figures given above the carbon

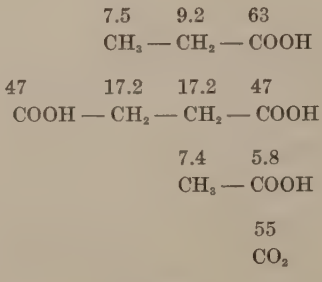


Figure 2

groups are the specific activity in percentage of the specific activity of the 3,4-carbons of the glucose. The fermentation was conducted with resting cells under nitrogen and the carbon dioxide evolved during the fermentation was trapped in alkali. It can be seen that approximately 8% of the 3,4-carbons appears in the ethylene carbons of the propionate, a distribution not predicted by either the glycolytic scheme or the *L. mesenteroides* mechanism. Even more striking is the

amount of the 3,4-carbons appearing in the ethylene carbons of the succinate, the radioactivity being very close to twice that of the ethylene carbons of the propionate. This labeling suggests that at least 8% of the propionate and 17% of the succinate were formed by some mechanism other than that of *L. mesenteroides* or by the glycolytic scheme. Moreover, since the ethylene carbons of the propionate have a considerably lower labeling than the ethylene carbons of the succinate, it is probable that not all the propionate could have arisen via succinate. This conclusion is reached on the basis that, if succinate were the precursor of propionate, the propionate would be expected to reflect the labeling of the succinate. This conclusion is valid only if the isotope distributions in the propionate and the succinate were constant throughout the fermentations or if there were a constant proportion of the succinate decarboxylated to propionate throughout the fermentation. If there were a change in the mechanism during the latter part of the fermentation so that the succinate formed had higher activity than that formed in the early fermentation, then it would be possible for the total propionate to be of lower activity than the succinate, even though all the propionate were formed from succinate. For example the succinate isolated from the fermentation might be representative of that formed at the end, rather than that formed earlier, and from which most of the propionate was formed. This possibility is being investigated.

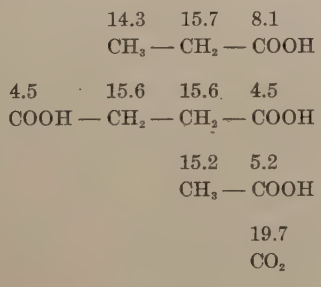


Figure 3

The distribution of labeled carbon in the end products obtained from glucose-1-C^{14} is shown in figure 3. The conditions of this fermentation were the same as those with glucose-3,4-C^{14}. Here again the activity is distributed into all the products. It will be recalled that methyl-labeled lactate gave a very similar distribution [equation (2c)]. Since the specific activity of the carbon dioxide is considerably higher than that of any of the carboxyl carbons of the end products, it does not seem likely that this carbon dioxide arose by decarboxylation of the acid percursors. The carbon dioxide appears to have arisen in part by a selective oxidation of the 1-carbon of glucose to carbon dioxide such as would be expected by the hexose monophosphate shunt.

In summary then these points must be borne in mind:

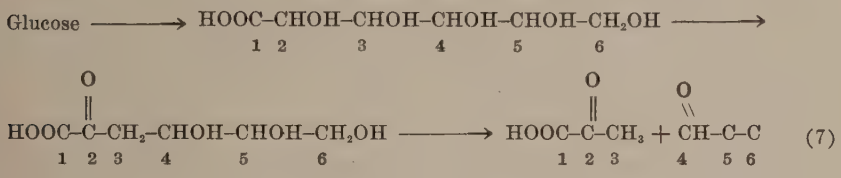
1. That carbon dioxide has a low turnover.
2. That formaldehyde seems to be an active intermediate during the dissimilation of glycerol and probably other substrates as well.
3. That the dissimilation of labeled glucose results in a C^{14} distribution in the end products not accounted for by the traditional glycolytic cleavage or the hexose monophosphate shunt.
4. That probably not all the propionate is derived via succinate.
5. That there is almost complete randomization of tracer in the α and β carbons of propionate, which may be an indication that it is formed from some symmetrical C_4 dicarboxylic acid.

These observations have led us to believe that some hitherto unexpected mechanisms of fermentation exist. One possible explanation of the results would be a split of the glucose by terminal cleavage of the 1- and 6-carbons to yield directly a C_4 acid from the glucose [equation (6)]. This type of cleavage would account for the high activity of the carbon dioxide

$$\begin{array}{l} \overset{1}{C}\!:\!\!-\!\overset{2}{C}\!-\!\overset{3}{C}\!-\!\overset{4}{C}\!-\!\overset{5}{C}\!-\!:\!\overset{6}{C} \longrightarrow \\ \underset{1}{CO_2} + \underset{2}{C}\!-\!\underset{3}{C}\!-\!\underset{4}{C}\!-\!\underset{5}{C} + \underset{6}{HCHO} \end{array} \qquad (6)$$

from 1-labeled glucose and the high labeling of succinate in the ethylene positions from 3,4-labeled glucose. In addition, the fact that fluoride inhibits succinate formation almost completely from C_3 compounds (Wood and Werkman, '40) but not from glucose might be explained as a direct formation of succinate by a nonfluoride-sensitive mechanism.

A more likely explanation may be the type of split found by Entners and Doudoroff ('52). With an enzyme preparation of *Pseudomonas,* they found that the carboxyl carbon of pyruvate arose from the number 1 carbon of the glucose. Presumably this cleavage might occur as shown in equation (7). This would be a cleavage similar to that found by Racker

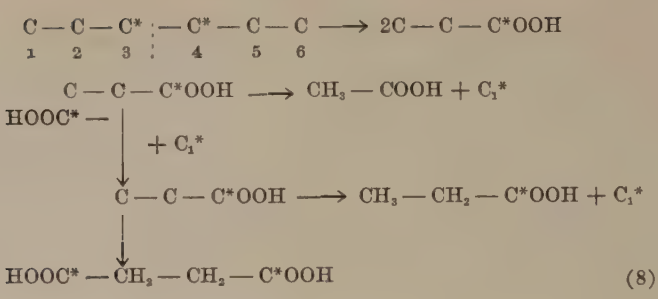


(7)

('52) in which desoxypentose is cleaved to acetaldehyde and a triose.

In our fermentations, if this were occurring it would seem necessary to assume that there was a preferential conversion of the pyruvate to succinate perhaps by the Krebs cycle, and of the triose to propionate. This would account for the higher labeling of the α and β carbons of the succinate than of the propionate when glucose-3,4-C^{14} was fermented. Also from 1-labeled glucose it would account for the preferential conversion of the 1-carbon of glucose to carbon dioxide.

Along with this conversion it would seem likely that the typical Meyerhof reactions would occur, which would yield carboxyl-labeled acids from the 3,4-labeled glucose and α,β-labeled acids from the 1-labeled glucose [equation (8)]. Thus from the desoxy and the Meyerhof types of split, one would obtain just the opposite types of labeling in the products, and the relative distribution in the products would depend on the relative occurrence of the two types of cleavage.



(8)

If this were the case it would seem likely that the relative occurrence of the two types of cleavage might be altered by use of fluoride, which would presumably block the Meyerhof type of cleavage. If the desoxy type of cleavage occurred in the presence of fluoride, then carboxyl-labeled pyruvate should be formed from 1-labeled glucose. However, preliminary experiments have not indicated any appreciable change in the distribution of C^{14} in the end products from fermentation of labeled glucose in the presence of 0.015 *M* sodium fluoride. Before too great a significance is attached to these results it will be necessary to show that the breakdown of PGA is inhibited by fluoride under the particular conditions used in this experiment.

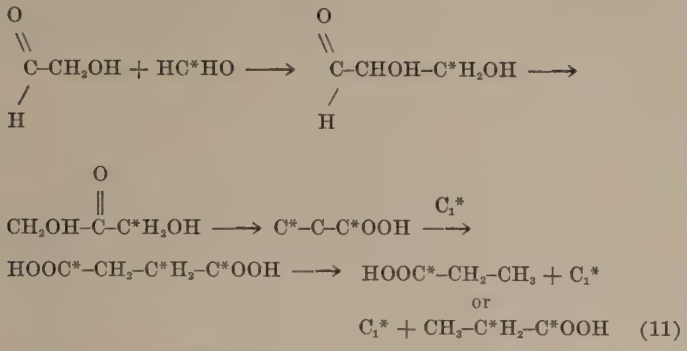
The mechanism whereby formaldehyde is formed from substrates and is utilized is not known. It is known that formaldehyde and glycine are in some way combined to form serine in animals [equation (9)]. A similar reaction might occur with glycolaldehyde or a triose [equation (10)].

$$\text{“HC*OOH”} + CH_2NH_2\text{–COOH} \longrightarrow C^*H_2OH\text{–}CHNH_2\text{–COOH} \quad (9)$$

$$HC^*HO + CH_2OH\text{–CHO} \rightleftharpoons C^*H_2OH\text{–CHOH–CHO} \quad (10)$$

The mechanism whereby added C^{14} formaldehyde is converted to all positions of propionate and succinate is also of great interest. Here again no definite information is available. One possibility is that formaldehyde is fixed in a symmetrical compound such as glycerol or dihydroxyacetone. If this occurred and propionate were formed from a C_4 dicarboxylic

acid by addition of a 1-carbon compound formed from formaldehyde, a distribution such as found in the propionate



would be observed [see equation (11)]. It is noted that, in this case, the C^{14} concentration would be twice as high in the carboxyl as it would be in the α or β position and the concentration of C^{14} would be equal in the α and β positions. This is approximately the distribution of C^{14} from formaldehyde that was observed in the propionate. The higher labeling found in the succinate would not be accounted for, however.

The occurrence of a symmetrical C_3 intermediate may also enter into the extensive randomization of the isotope in the end products from the labeled sugars. For example, if 3,4-labeled glucose were split in part to dihydroxyacetone phosphate and the phosphate removed to yield a symmetrical C_3, it is clear from the preceding remarks that the isotope would not reside entirely in the carboxyl groups, even though the initial cleavage was a typical glycolytic split. Thus it is clear that it is not necessary to have different types of cleavage to obtain the observed extensive randomization of the tracer in the end products. The facts that there are fluoride- and nonfluoride-sensitive mechanisms and that the 1-labeled glucose yields carbon dioxide with a higher C^{14} concentration than the carboxyls of the end products lead one to suspect that the randomization does occur in part because of simultaneous

occurrence of more than one mechanism. It is not unlikely that, superimposed on the different mechanisms, there may be additional randomization due to occurrence of a symmetrical C_3. This may, in part, account for the fact that sodium fluoride does not prevent the randomization of C^{14} from the different types of labeled sugars.

It is clear that, unlike some of the other studies on labeled sugars with bacteria, the picture with the propionic acid bacteria is very complex. It will therefore be necessary to limit the reactions, perhaps by fractionation, of the ezymes before a clear understanding can be obtained of the over-all complicated picture of the fermentation. It is realized that much that has been said is speculative, but at present it seems necessary that the explanations be incomplete and tentative.

DISCUSSION

BARD: Some years ago Doctor Gunsalus and I made the unfortunate discovery that *Clostridium* is capable of a vigorous respiration of glucose as a substrate. We spent a great deal of time studying this, with very few results that we can talk about.

But one observation which Doctor Leaver just discussed, namely, the insensitivity to fluoride, strikes a reminiscent note. I remember the data quite incompletely, but I do recall that, although fluoride inhibited oxygen uptake to a considerable extent, decarboxylation proceeded nevertheless.

LEAVER: There has been a fair amount of study reported in the literature of the inhibition by the fluoride of the propionic acid fermentation. It has been found when fluoride is added to a culture fermenting glucose that one obtains the same end products with or without fluoride.

Likewise, cells grown in the presence of sodium fluoride and washed free of it will not ferment PGA. In fact, a cell-free extract can be made and the cell-free extract will not ferment PGA, whereas it will ferment glucose. From what is known of a typical Meyerhof scheme, it seems that PGA

utilization is stopped and is not involved when glucose is fermented in the presence of fluoride.

H. G. Wood: It interests me that fluoride has been shown to inhibit succinate formation and net CO_2 fixation from a number of substrates by the propionic acid bacteria; and that fluoride stopped succinate formation almost quantitatively from 3-carbon compounds such as glycerol and pyruvate. But in the fermentation of glucose with fluoride present, the inhibition of the formation of succinate was not nearly so complete. The amount can be reduced but it is never cut down to zero. This made us wonder if possibly the succinate were coming directly from the sugar, or at least by some mechanism other than carbon dioxide utilization.

Gibbs: I think the formaldehyde that is made here at Oak Ridge National Laboratory contains a fair amount of labeled methanol. I was wondering how this would complicate the picture. Were controls perhaps run with methanol?

Leaver: We made the formaldehyde and did check for the presence of methanol; there was a trace amount. Most of the methanol had been removed by passing the vapor through calcium chloride. However, the experiments were run in such a manner that practically a C^{14} balance was obtained, and the trace amount of methanol — less than 1% — would not change our observations.

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THE BIOLOGICAL FUNCTIONS OF PTERIDINE DERIVATIVES

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FOUR FIGURES

The pyrimido-pyrazines for which the name "pteridine" was suggested by Wieland (Schopf et al., '41) are of very widespread occurrence in nature. Simpler derivatives such as leucopterin, xanthopterin, and isoxanthopterin are found in butterflies (Purrmann, '45), certain insects (Schopf and Becker, '36), in human urine (Koschara, '36), and probably in bacteria (Crowe and Walker, '44). More complex derivatives such as the pteroyl glutamates and "citrovorum factor" (folinic acid?) are indicated by biological assay to be constituents of almost all living cells that have been examined.

The problem of the biological function of pteroylglutamic (folic) acid and related compounds has received much attention in the past decade. An overwhelming amount of evidence has accumulated, indicating that a major function of this vitamin is related to the synthesis of thymine and the purine constituents of nucleic acids. The specific part played by folic acid in the synthesis of purine appears to be the insertion of carbon-2 and perhaps also carbon-8 into the purine ring. Since purine carbons-2 and -8 and likewise the 5-methyl group of thymine may be derived from administered formic acid (Buchanan et al., '48; Totter et al., '51), the role of folic acid appears to be the metabolic transfer and/or the metabolic formation of an active 1-carbon "fragment" often loosely called "formate." In addition, certain reactions involving labile methyl formation and transfer appear to be affected

by *p*-aminobenzoic acid, a constituent of folic acid, in bacteria (Kohn, '43; Shive and Roberts, '45), by folic acid in animals (Bennett, '49), and by the functionally related vitamin B_{12} in both bacteria and animals (see reviews by Jukes and Stokstad, '51; Shive, '50). It may be supposed, therefore, that the function of folic acid and its derivatives may be largely explained on the basis that it is concerned with formation and/or transfer of 1-carbon fragments in such a manner that all 1-carbon intermediates in any state of oxidation below carbonic acid are affected.

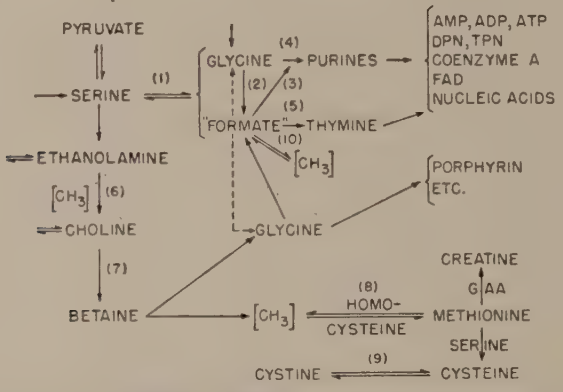

Fig. 1 Summary of reactions related to 1-carbon transfers. GAA = guanidoacetic acid. FAD = flavinadeninedinucleotide. AMP, ADP, ATP = adenosine-mono-, -di-, -triphosphate.

The studies leading to the conclusions reached have been so well reviewed in recent publications (Welch and Heinle, '51; Shive, '50, '51) that it seems superfluous to give more than a very brief summary here. The present paper, therefore, will deal largely with those aspects which have received but little attention in the past few years. An attempt will be made to delineate what effects may be attributed to a primary function of folic acid and what may well be due to secondary deficiencies related to a lack of the vitamin.

Some of the metabolic interrelationships between sources of the 1-carbon intermediates and purines, thymine, coen-

zymes, amino acids, etc., are shown in abbreviated form in figure 1. The rates of many of the reactions shown in this diagram may be affected by a deficiency of folic acid. In particular, reactions (1), (2), (3), (4), (7), and (8) have been demonstrated to be altered by simple dietary- or antagonist-induced folic acid deficiency (see reviews by Shive, '51; Jukes and Stokstad, '51).

The close relationship of most of the reactions in figure 1 to 1-carbon transfers, whether of methyl groups or of "formate," has led to a widespread belief that a coenzyme form of folic acid, possibly citrovorum factor, acts by accepting and donating formyl groups in a general manner. This hypothesis has found much support in the investigations of Shive and collaborators at Texas, as well as many others. The subject has been completely covered in the recent reviews of Shive ('51) and Welch and Heinle ('51). Recently Buchanan ('51) has found that citrovorum factor stimulates an interchange of carbon-2 of inosinic acid with radioactive formate.

The interpretation of the action of folic acid as the precursor of a general transformylating coenzyme chiefly concerned with purine and thymine production can explain most of the phenomena associated with the various types of folic acid deficiencies. However, some experimental findings are reconciled by this hypothesis only with considerable strain. Of special interest is the fact that the rhesus monkey requires about 0.1 mg of folic acid per 100 gm of diet (Day and Totter, '47) while the requirement for the growing chick is usually set between 0.04–0.1 mg per 100 gm of diet (Keith et al., '48). The purine production of the monkey, as indicated by allantoin and uric acid excretion, accounts for less than 1% (0.6%) of dietary nitrogen (Dinning and Day, '49) whereas the chick excretes the major portion of its dietary nitrogen as purine nitrogen. This discrepancy indicates that there is either an enormous difference in the efficiencies of transformylations involving purine production or that purine production is a quantitatively minor function of the co-

enzyme involved. The folic acid-deficient chick continues to excrete uric acid at a rate more than sufficient to supply its expected nucleic acid and purine requirement.

Folic acid would be unique among the B vitamins if its function turned out to be quite different in the chick as compared with the mammal. Likewise it appears improbable that the synthesis of purines is wholly different in the chick and in the mammal.

Another experimental finding which is difficult to reconcile with a transformylating function for folic acid in which the formyl group is required to be actually attached to a nitrogen atom as in the citrovorum factor (folinic acid?) (Pohland et al., '51) is that certain steroids (dehydroisoandrosterone, cortisone) can replace folic acid in the nutrition of microorganisms (Gaines and Totter, '50; Gaines et al., '51). It has been found that the steroid stimulates the utilization of radioactive formate by *Streptococcus faecalis* just as does folic acid (Totter, '52). It might be assumed that steroids owe their activity to an ability to stimulate the bacterium to synthesize its own folic acid. If this is so, it must also be assumed that steroids can overcome the block to folic acid synthesis imposed by sulfanilamide, since growth is stimuated in *Escherichia coli* in the presence of the latter compound (Gaines and Totter, '50).

Many other functions not obviously related to the "formate" transformations indicated in figure 1 have been suggested for folic acid and its derivatives. Among these may be mentioned the effect of folic acid on tyrosine metabolism in man (Swendseid et al., '47) and in the scorbutic guinea pig (Woodruff and Darby, '49). Davis ('46) believes that folic acid and B_{12} are related to the cholinesterases. Martin and Beiler ('47) have presented data which suggest that mammalian kidney dopadecarboxylase requires folic acid for its activity. Dinning and co-workers ('49, '50) have suggested a relation of folic acid to choline oxidase. Williams ('51) has further studied this possible relationship.

The observation by Kalckar et al. ('48; see also Wieland and Liebig, '44) that certain pteridine derivatives are powerful inhibitors of xanthine oxidase deserves greater consideration than has hitherto been given. The demonstration by the Arkansas group (Keith et al., '48) that folic acid deficiency in the chick leads to a considerable elevation in xanthine oxidase of the liver has been amply confirmed in other laboratories (Williams et al., '49; Remy and Westerfeld, '51), but interpretation of the importance of the phenomenon has been lacking. The studies of Greenberg on the mechanism of formation of hypoxanthine in pigeon liver suggest that a reinterpretation of the significance of the elevated xanthine oxidase is in order.

According to Greenberg ('51), the formation of hypoxanthine in pigeon liver may be represented by the following series of reactions:

$$\text{Glycine} + 3\ NH_3 + 2\ \text{``formate''} + CO_2 + \text{ribose phosphate} \longrightarrow \text{inosine-5-phosphate} \quad (1)$$

$$\text{Inosine-5-phosphate} \longrightarrow \text{inosine} + H_3PO_4 \quad (2)$$

$$\text{Inosine} + H_3PO_4 \longrightarrow \text{hypoxanthine} + \text{ribose-1-phosphate} \quad (3)$$

To these may be added the oxidation of hypoxanthine to uric acid wherever xanthine oxidase is also present, as in chick liver. The latter reaction is probably not metabolically reversible to any considerable degree. An elevated xanthine oxidase would, therefore, constitute a perpetual drain on the inosinic acid which might otherwise be utilized for nucleic acid or coenzyme formation.

An argument against this supposition is the fact that rats rendered deficient in folic acid have not been shown to have an unequivocally elevated xanthine oxidase (Schmitt and Petering, '49). However, it should be remembered that certain pteridines are xanthine oxidase substrates which are also competitive inhibitors for the oxidation of purines (Lowry et al., '49). When high concentrations of substrate are utilized for determination of xanthine oxidase, any naturally present inhibitor may be displaced. That this may actually be the case is indicated by the results shown in figure 2. In this

experiment liver homogenates from succinylsulfathiazole-fed rats were used. The xanthine oxidase was determined at 25°C. in the presence of 0.5 μM per milliliter of hypoxanthine by observing the change in optical density at a wave length of 290 mμ, as in the method of Kalckar ('47). The homogenates were centrifuged to remove uricase. In the method of Axelrod and Elvehjem ('41) used by Schmitt and Petering ('49) the molarity of the substrate was about 7 times this

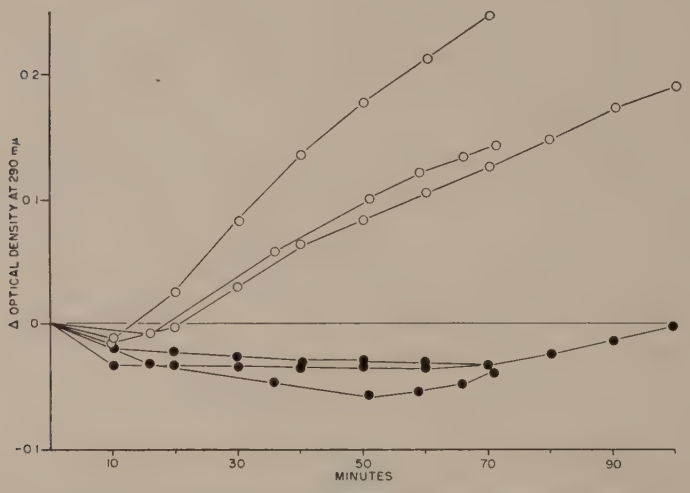

Fig. 2 Xanthine oxidase in livers of succinylsulfathiazole-treated rats. Animals fed 4–5 weeks on 2% succinylsulfathiazole in purified diet. 1 ml of 1: 80 liver homogenate, 0.2 mg hypoxanthine, 1 ml 0.05 M phosphate buffer, pH 8. Total volume, 3 ml. Optical density determined on Beckmann spectrophotometer. ● = control. ○ = folic acid deficient.

high. Westerfeld and Richert ('50) used a similar substrate concentration. These latter investigators failed to find any effect of folic acid on the liver xanthine oxidase of rats. It may be seen that, under conditions of low substrate concentration, striking differences can be found. The results may indicate that the amount of xanthine oxidase available for purine oxidation is low in control animals, the remainder being inhibited by a pteridine derivative, the level of which controls the rate of purine destruction by a competitive mecha-

nism. It may be seen from figure 2 that the control liver homogenates do begin to oxidize hypoxanthine after an induction period of about one hour.

That the synthesis of purine or thymine from formate is not markedly impaired in the folic acid-deficient chick was shown by Totter, Volkin, and Carter ('51). In further ex-

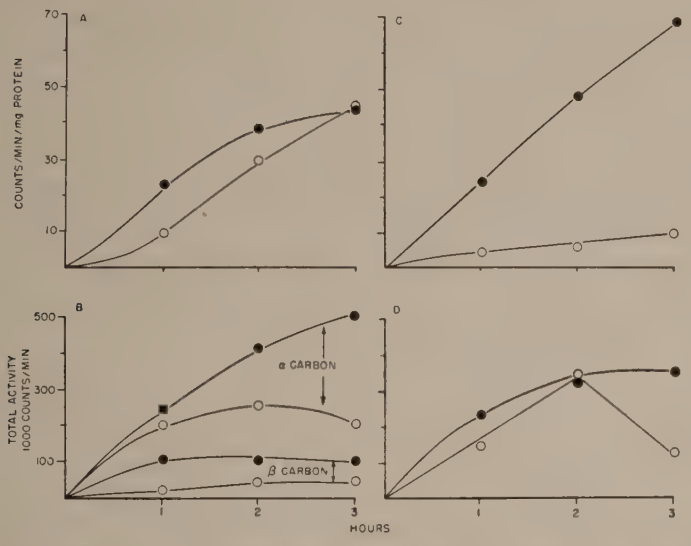

Fig. 3 Uptake of C^{14} formate and 2-C^{14}-glycine by control and folic acid-deficient chick liver slices. 1 gm of liver suspended in 2 ml beef serum ultrafiltrate. 0.2 ml containing 5 μM (20 μc) of formate or 0.5 ml glycine-2-C^{14} (24.2 μM, 22.5 μc) as substrate. ● = control. ○ = folic acid deficient.

Substrate: $C^{14}H_2NH_2COOH$ — A = protein counts
B = serine counts

Substrate: $HC^{14}OONa$ — C = protein counts
D = serine counts (β carbon)

periments by Carter and the author conducted in the Biology Division at Oak Ridge, the rates of conversion of formate to serine and glycine to serine were studied with C^{14}-labeled compounds, using control and folic acid-deficient chick liver slices. Some of the data given in figure 3 show that the rate of incorporation of formate into serine was not seriously diminished by folic acid deficiency. However, the incorpora-

tion of the serine into the liver protein was almost abolished. This may help to explain some of the seeming discrepancy between our results and those of Plaut and collaborators (Plaut et al., '50) who found tissue serine much less rapidly labeled in their succinylsulfathiazole-fed rats untreated with folic acid, as compared with treated controls.

E. Popp (unpublished experiments), in our laboratory, has investigated the radioactivity of the uric acid from the liver slice experiments. He has found that, although at the end of the first hour there was less activity in uric acid in the slices from deficient chicks, the two- and three-hour samples showed much higher activities than did the controls.

In contrast to the ability of the deficient-chick liver slices to incorporate formate rapidly into serine, glycine was found to cleave slowly. From figure 3 it can be seen that when 2 C^{14}-glycine was the external source of "formate" only a small proportion of the β carbon of the isolated serine was active. On the other hand the slices from folic acid-fed chicks rapidly incorporated the methylene carbon of glycine into the β carbon of serine. Similar experiments need to be performed with labile methyl as precursor of "formate" to determine whether a block to purine synthesis in simple folic acid deficiency is in the formation rather than in the utilization of "formate."

Skipper and collaborators ('50) have shown that formate utilization for purine synthesis is impaired in aminopterin-treated mice. Whether this treatment is equivalent to simple dietary folic acid deficiency remains to be shown.

A reduced supply of purine derivatives, particularly of adenylic acid, whether brought about by failure of synthesis or by too rapid destruction would inevitably lead to profound alterations in tissue metabolism. While effects of the impaired nucleic acid synthesis have been emphasized by almost every investigator in the field of folic acid and B_{12} function, it is quite likely that there are other alterations which are even more significant.

The importance of adenylic acid and its derivatives for glycolysis and other metabolic transformations is well known. Further, adenylic acid is a constituent of several coenzymes, notably disphosphopyridinenucleotide, triphosphopyridinenucleotide, flavinadeninenucleotide and coenzyme A (Beinert et al., '52; Gregory et al., '52). It would appear that a reduction in synthesis of adenylic acid must inevitably lead to a disturbance in formation of these coenzymes which might well be reflected by a loss of function characteristic of a dietary deficiency of the vitamin involved.

Examination of this idea leads one to the conclusion that the degree of deficiency manifested might differ in different animals, in each organ of an experimental animal, and for each of the vitamins. The level of coenzyme present in each tissue is dependent upon several factors aside from the concentration of the vitamin itself and of adenylic acid. The route of synthesis and destruction and the equilibrium constants of the reactions are of importance as well as the influence of other reactions requiring adenylic acid or adenosinetriphosphate (ATP) and which would be competing with coenzyme formation.

In 1950 there became available an extremely sensitive method for ATP (Strehler and Totter, '52) based on the discovery by McElroy ('47) that ATP restored the luminescence of firefly lantern extracts after its exhaustion. By appropriate modification this method can be used for assay of many enzymes and substrates which are related to energy-transfer mechanisms. For instance, by using a creatine transphosphorylase preparation (Lohman, '34) and phosphocreatine it is possible to detect minute amounts of adenylic acid. The method has been used to study the apparent free adenylic acid content (after disappearance of ATP) in tissues of folic acid-deficient chicks. It has been found, even though there is some variability in results, that the free adenylic acid content of the heart (and less frequently of the breast muscle) of deficient chicks is often very low compared with that of folic acid-fed controls. Figure 4 indicates the great differ-

ence in rate of resynthesis of ATP occasioned by the lower adenylic acid content of the breast muscle from a deficient chick. In this case the transphosphorylase used was that of the muscle itself. An independent determination indicated that the enzyme was also somewhat low in the deficient chick,

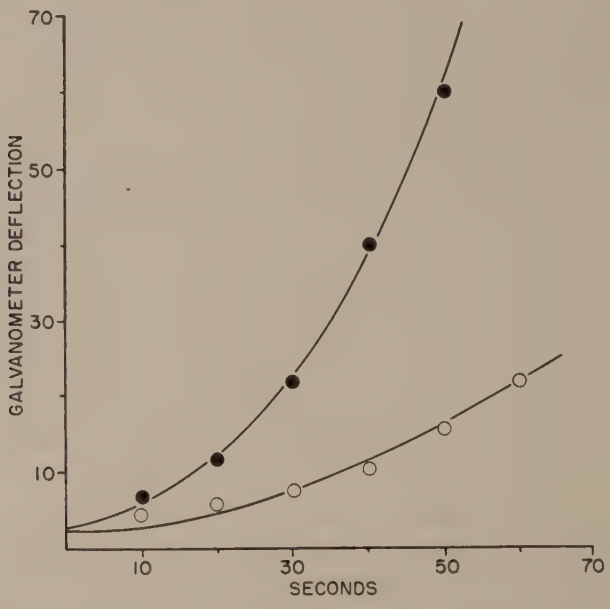

Fig. 4 Adenylic acid determination by adenosinetriphosphate resynthesis. Control and folic acid-deficient chick breast muscle. 0.2 ml of 1:100 firefly lantern extract in 0.1 *M* arsenate buffer, pH 7.4; 2 mg $MgSO_4 \cdot 7H_2O$; homogenate of 10 mg muscle; 200 μg phosphocreatine; total volume, 0.8 ml. Full scale deflection (100) is equivalent to approximately 5 μg adenylic acid. ● = control. ○ = folic acid deficient.

but the major part of the difference shown is due to lack of adenylic acid in the deficient chick muscle.

There are obvious possible applications of these findings to the explanation of various clinical and experimental observations. When consideration is taken of the possibility that folic acid deficiency leads to a multiple vitamin-B deficiency, apparent similarities between functions and overlapping activities are much less confusing.

The literature indicates that signs of other B-vitamin deficiencies are abundantly evident in folic acid- or B_{12}-deficient animals. Several years ago Wright and Welch ('44) called attention to the apparent pantothenic acid deficiency seen in succinylsulfathiazole-fed rats and preventable with folic acid but not with excess pantothenic acid. In the livers of folic acid-deficient animals, pantothenic acid (now known to occur in liver almost wholly in the coenzyme form) remained low regardless of the dietary level. A relationship between vitamin B_{12} and pantothenic acid has been found by Yacowitz, Norris, and Heuser ('51).

Daft and collaborators ('45) studied pantothenic acid deficiency in rats and found leucopenia and granulopenia, signs which are "characteristic" of folic acid deficiency. Some of their animals responded to folic acid and some to pantothenic acid alone, while better results were obtained when folic acid was given together with pantothenic acid.

Ruegamer and co-workers ('48) found that dogs recovered more quickly and more surely from nicotinic acid deficiency when folic acid and liver extract were simultaneously administered. The Wisconsin investigators have used riboflavin deficiency to induce a deficiency of the monkey "antianemia factor" (Cooperman et al., '45). Their results resemble somewhat those of Daft et al. Pernicious anemia in man, which is related to both folic acid and vitamin B_{12}, has been frequently likened by clinicians to the nicotinic acid-deficiency disease, pellagra. Several signs and symptoms are the same in both diseases. Table 1 gives a summary of some of the similarities between various vitamin deficiencies that have been encountered. The information in this table could be greatly amplified.

The evidence seems to indicate a relation of the coenzyme forms of pantothenic and nicotinic acids to the function of folic acid. The more complex relation of riboflavin coenzymes has been discussed briefly but requires much further experimental elucidation. It is also possible that vitamins such as thiamine and pyridoxal are dependent for their maintenance in

TABLE 1

Signs associated with syndromes related to deficiencies of various members of the B complex

	NIACIN DEFICIENCY		PANTOTHENIC ACID DEFICIENCY	FOLIC ACID DEFICIENCY		PERNICIOUS ANEMIA	SPRUE
	Man	Dog	Rat	Monkey	Rat		
Leucopenia	?	?	+	++	++	+	+
Anemia	+Macrocytic	+Macrocytic	+	+	+	++	++
Bone marrow	?	Megaloblastic	Hypoplastic	Megaloblastic		Megaloblastic	Megaloblastic
Intestine	Lesions		Gastroenteritis	Lesions			Lesions
Diarrhea	++	+	+	++	+?	—?	++
Pigmentation			Abnormal		Abnormal	Abnormal±	Abnormal
Nervous system damage	++	++	+	?	?	+	++

\+ Indicates that the condition is frequently observed.
++ Indicates that the alteration is characteristic of the syndrome.

coenzyme form upon normal levels of tissue adenine derivatives.

With an elevated liver xanthine oxidase and the probable secondary effects related to lack of adequate adenylic acid, it is possible to explain most and perhaps all of the folic acid deficiency signs occurring in at least one experimental animal, namely the chick, without recourse to a transformylating function for the vitamin. However, if folic acid does not provide the transformylating coenzyme directly it must affect it indirectly. It is possible that coenzyme A (CoA) may be involved as a general acylating coenzyme. The cleavage of serine and glycine to yield a 1-carbon intermediate somewhat resembles the CoA-catalyzed formation of formate from pyruvate in *E. coli* (Kalnitsky and Werkman, '43). These possibilities are under investigation in our laboratory at the present time.

In this connection, the requirement of birds for pantothenic acid — the precursor of CoA — is higher than that for the rat, a finding unlike that for folic acid, discussed here.

The extreme view that pteridine derivatives do not have a transformylating function is probably not justifiable, but has been developed here for the purpose of pointing out that important functions for pteridines may have been overlooked because of too much emphasis on one avenue of approach.

Structural considerations have been used to support the proposal for a transformylating function for citrovorum factor (folinic acid?). Similar considerations would justify the suggestion that pteroylglutamic acid and its derivatives might play a role in "transglutamylation." One of the most promising leads of recent years appears to be the discovery by Bakerman et al. ('51) that succinylsulfathiazole-induced folic acid deficiency in the rat leads to a profound disturbance in glutamic acid metabolism characterized by an excessive excretion of this amino acid. The further studies of this group are awaited with interest.

Other naturally occurring pteridines may have functions perhaps unrelated to those discussed. Koschara ('40) has

isolated from urine a sulfur-containing pteridine, urothione. This compound apparently contains a methylthiol group. Such a compound could well be an intermediate for transmethylation. Whether folic acid is the biological precursor of this substance is unknown, but it is well known that folic acid affects transmethylation, either directly or indirectly (see review by Welch and Heinle, '51).

The isolation from fireflies *(Photinus pyralis)* of another compound of considerable interest was recently reported by Strehler ('51). The analysis, fluorescence, reactions, and absorption spectrum of this compound, luciferesceine, strongly suggest that it is a pteridine derivative, perhaps intermediate in structure between riboflavin and the simpler pteridines. The close association of luciferesceine with luciferin and certain similarities in fluorescence and absorption spectrum (Strehler, personal communication) suggest a chemical relationship between the two. Should this prove to be true, a function for the pteridines in the luminescence reactions may be added to those already discussed.

SUMMARY

In summary, the widely held theory that pteroylglutamic (folic) acid or its coenzyme form acts as a cotransformylase was briefly discussed. An alternative mechanism to explain some of the interference with purine synthesis observed in folic acid deficiency states was proposed.

It was suggested that a large proportion of the signs and symptoms of folic acid deficiency are attributable to secondary deficiencies of coenzymes, especially coenzyme A, occasioned by a lowered tissue adenylic acid concentration.

DISCUSSION

KELLY: Doctor Totter, do you know of any measurements on nucleic acid turnover which indicate an effect of folic acid in normal animals?

TOTTER: I know of none, but I do know that in microorganisms, if one of the sources of formate, such as serine or gly-

cine, is increased and folic acid added, a strong inhibition of growth is obtained. This inhibition can be reversed by folic acid antagonists.

H. G. Wood: I am sure that some of you would be interested in an observation that Berg has made in our laboratory on the conversion of formate to the methyl group of methionine. He used an extract of pigeon liver and added homocysteine to act as a methyl acceptor in the conversion of the C^{14} to the methyl group. In the course of his work, he also determined the total amount of formate used in the system, and found that much more formate was being used than was being converted to methionine. This led Berg and Greenberg to carry out further studies, and it is now quite clear that homocysteine is involved in the insertion of formate into the 2 and 8 positions of purines and conversion to the β carbon of serine as well as to the methyl of methionine. The requirement for homocysteine is specific in that cysteine and glutathione will not replace the homocysteine.

The possibility that serine and cystathionine are intermediates in the conversion of formate to the methyl carbon of methionine appears unlikely on the following basis.

1. The disparity of specific activities with C^{14} formate, i.e., serine>methionine>cystathionine.

2. The low dilution of the C^{14} incorporated into methionine when pools of unlabeled serine or cystathionine are present.

3. The fact that serine is converted to methionine at one-third the rate of formate, and cystathionine is not converted at all.

It seems possible that the homocysteine forms some type of compound with formate, possbily RS-CHO. This RS-CHO may serve as a common precursor for the incorporation of formate to methyl, to serine, and to purine. This suggestion is supported by the fact that stimulation of any one of the pathways may be brought about by adding acceptors to shunt the isotope in the different directions. Thus if glycine is added, incorporation of formate into methyl carbons is decreased, whereas incorporation in the β carbon of serine is

markedly increased. Similarly, if glycine, bicarbonate, glutamine, and ATP are added, the incorporation in purines is high, while that in serine and in methyl groups is decreased.

If homocysteine is a formyl acceptor, as these results seem to indicate, one is led to wonder if folic acid functions as a formyl carrier to the homocysteine or whether its real function is in some other capacity.

Balis: Doctor Totter has mentioned the fact that folic acid does more than merely control the synthesis of purines and thymine. The behavior of *L. casei* tends to confirm this. The organism is capable of incorporating formate into the purines of the pentose nucleic acid in the presence of folic acid and not in the absence of folic acid. However, no amount of purine and thymine added to the medium in place of folic acid can restore growth to the full extent obtained with folic acid.

Stadtman: We have made one attempt to get the formation of CoA formate by the CoA-transphorase reaction. In this experiment we used acetyl phosphate as the acetyl donor rather than acetyl-CoA and looked for the disappearance of acetyl phosphate in the presence of the formate and for the formation of something that might be considered a phosphoryl derivative of formate. This experiment was completely negative.

H. G. Wood: My knowledge of this is limited but, as I understand it, Silverman of the National Institutes of Health has now found that — in folic acid deficiency — when glutamic acid is excreted, it comes from histidine. Apparently, a formyl acceptor is required to split it, and the ureide carbon drops out of the histidine in the transfer. This story may not be quite complete, but those are essentially the facts; when histidine is given, a greatly increased excretion of glutamic acid is obtained.

Carter: Is that isoglutamine?

Wood: It is not glutamic acid; it is in the form of formyl isoglutamine.

Carter: Again homocysteine would be a good acceptor.

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PURINE AND PYRIMIDINE METABOLISM IN MICROORGANISMS [1]

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FIVE FIGURES

Interest in the metabolism of purine and pyrimidine compounds in microorganisms stems from many stimuli. Certainly, the discovery of the desoxyribonucleic acid-activated transformation of pneumococcal types by Avery et al. ('44) has been one of the most overwhelming influences in directing and accelerating research toward a better understanding of microbial nucleic acid synthesis and function.

The studies of the coenzymes that have purine and pyrimidine units within their structural makeup, the studies of bacteriophage synthesis, the studies of growth responses by microbiological systems to nucleic acid intermediates and related studies of growth inhibition by antimetabolites, the studies of metabolic pathways by use of the mutant and isotopic tracer techniques, the histochemical studies of nucleic acid distribution within cells, the chemical studies on the isolation and analysis of nucleic acids — all these varied and important approaches are contributing to our knowledge of purine and the pyrimidine metabolism.

In this review of purine and pyrimidine metabolism the enzymatic method will be stressed on the premise that the existence of many important intermediates can be demonstrated only after the detection and purification of the enzymes involved in a series of metabolic conversions.[2]

[1] This investigation was supported by a research grant from the National Institutes of Health, U. S. Public Health Service.

[2] A very excellent review of purine and pyrimidine metabolism at the coenzyme level has recently appeared (Kornberg, '51).

The enzymatic activities to be discussed are classified as either "nucleolytic" or "nucleosynthetic" (table 1). The term "nucleolytic" has recently been introduced by Laskowski ('51) to describe various enzymatic irreversible hydrolyses of nucleic acid intermediates. This is an analogy to the term "proteolytic" as used in protein research. The so-called nucleosynthetic enzymes are those which catalyze readily reversible or energy-requiring reactions.

In setting up this classification it is fully realized that enzymatic activities which appear under one heading may change parties overnight. For example, the nucleosidases

TABLE 1

Enzymatic activities of microorganisms associated with purine and pyrimidine metabolism

Nucleolytic enzymes:	
	Desoxyribonuclease
	Ribonuclease
	Deaminases
	Phosphatases
	(Purine and pyrimidine dehydrogenases)
Nucleosynthetic enzymes:	
	Nucleoside phosphorylases
	Desoxyribosyl-*trans*-N-glycosidase
	Adenosine phosphokinase

were considered to be nucleolytic enzymes for many years. They were then found to be phosphorylases coming under the classification of nucleosynthetic enzymes. Recently some of the microbial nucleosidases thought to be phosphorylases have turned out to be old-fashioned nucleolytic enzymes. The dehydrogenases, involving a complexity of reactions, are included under the nucleolytic heading for convenience only.

At the present time very few nucleosynthetic enzymes are known. However, many more nucleosynthetic enzymes catalyzing amination and transamination reactions, group transfer reactions, and phosphorylation reactions, to name just a few, are certain to be characterized in the near future.

NUCLEOLYTIC ENZYMES

Desoxyribonuclease. Plenge ('03) described the liquefaction of calf thymus desoxyribonucleic acid (DNA) gels by a variety of bacteria. Iwanoff ('03) cultured *Aspergillus niger* and *Penicillium glaucum* in a medium containing calf thymus (DNA) as the only source of nitrogen and phosphorus. About 67% of the DNA nitrogen was converted to mycelium nitrogen; 30% of the DNA phosphorus was converted to mycelium phosphorus and 70% released as inorganic phosphate. Iwanoff prepared pastes and water extracts of *A. niger* by grinding the mycelium with kieselguhr. These enzyme preparations liquefied DNA gels and catalyzed a slow but definite release of inorganic phosphate.

McCarty and Avery ('46) found that pneumococcus type III cells, upon lysis with sodium desoxycholate, released desoxyribonuclease whose enzymatic behavior appeared to be similar to that of desoxyribonuclease from beef pancreas. The heat lability, optimum pH, dependence upon activation by magnesium ion, and destruction of the pneumococcal transforming substance were the same for the pneumococcal and pancreatic enzymes. Larger yields of transforming factor could be obtained by effectively inhibiting pneumococcal desoxyribonuclease with citrate.

Cohen ('47) demonstrated the depolymerization of T2 bacteriophage by desoxyribonuclease released from *Escherichia coli* cells lysed through phage action.

Tillett et al. ('48) and McCarty ('48) found that filtrates from cultures of hemolytic streptococci contained a potent desoxyribonuclease. This enzyme acted on DNA isolated from human pleural exudates and on calf thymus DNA. Streptococcal desoxyribonuclease was further purified (McCarty, '49; Christensen, '49).

McCarty ('49) made the interesting observation that, despite the fact that the purified streptococcal and pancreatic enzymes appeared to have the same enzymatic specificity, the antigenic specificities appeared to be quite different. Rabbit antisera prepared against the streptococcal enzyme inhibited

the action of the enzyme on its substrate, whereas the activity of pancreatic desoxyribonuclease was not affected. Antibody against the pancreatic enzyme did not inhibit the streptococcal preparation.

Bernheimer and Ruffier ('51) found that washed resting cells of streptococci elaborated extracellular desoxyribonuclease in the presence of glucose or maltose, or phosophate and magnesium ions. No enzyme was produced in the absence of any one of these factors.

Zamenhof and Chargaff ('49) described desoxyribonucleases prepared from yeast and from *Neurospora crassa*. The yeast enzyme attacked calf thymus and yeast DNA. Aqueous extracts of fresh crushed yeast cells exhibited practically no desoxyribonuclease activity. Upon prolonged standing at 4°C. the activity increased fiftyfold. The activated enzyme was further purified ninetyfold. The activation on standing could be explained by the proteolytic destruction of a labile protein which inhibited yeast desoxyribonuclease. Desoxyribonucleases from pancreas and *Neurospora* were not affected by the yeast protein inhibitor, whereas the yeast enzyme was markedly inhibited.

Zamenhof and Chargaff have postulated that the yeast inhibitor, through its control of desoxyribonuclease activity, may play an important role during critical periods of cell division.

Ribonuclease. MacFadyen ('34) found that suspensions of *Bacillus subtilis* cells degraded yeast ribonucleic acid without the release of inorganic phosphate. Up to that time there had been very little convincing evidence for the bacterial disintegration of ribonucleic acid other than the slow release of inorganic phosphate. MacFadyen found that after 48 hours about 80% of the nucleic acid was degraded to nucleotides not precipitable by uranyl chloride.

Woodward ('44) prepared cell-free extracts of an avirulent strain of *Pasteurella pestis* by sonic disintegration of the cells. These extracts depolymerized yeast nucleic acid to the oligonucleotide level with only a part being hydrolyzed to mono-

nucleotides. The decomposition was accompanied by liberation of only a trace of inorganic phosphate.

McCarty ('48) found that hemolytic streptococci produced ribonuclease as well as desoxyribonuclease in the culture medium.

Taka-diastase deaminase. Borsook and Dubnoff ('39) prepared a cell-free, partially purified extract of *Aspergillus wentii* which released ammonia from adenylic acid or nucleic acid but not from free adenine.

Mitchell and McElroy ('46) found that taka-diastase (prepared from water extracts of *Aspergillus oryzae)* was an excellent source of an enzyme catalyzing the conversion of adenosine to inosine. The taka-diastase deaminase was purified eightfold by further ethanol fractionation, representing a 600-fold purification over dry *Aspergillus* bran. Adenosine-3′-phosphate and adenosine-5′-phosphate were also deaminated, but this could be accounted for by the presence of phosphatase in the deaminase preparations.

Kaplan et al. ('52) have further purified the taka-diastase deaminase until a stage where their preparations are devoid of phosphatase activity. The surprising finding is that this enzyme can catalyze the direct deamination of a large number of adenine compounds. Adenine in the following combinations could be deaminated: 5-adenylic acid, adenylic acid *b* (3-adenylic acid ?), diphosphopyridinenucleotide (DPN), adenosinetriphosphate (ATP), adenosinediphosphate (ADP), and adenosinediphosphate ribose as well as adenosine. Adenine, triphosphopyridinenucleotide (TPN), and adenylic acid *a* (2-adenylic acid ?) are not attacked. The interesting possibility of a direct deamination of adenine while in combination as nucleic acid should be investigated with the purified enzyme. The nonspecificity of the taka-diastase deaminase is in marked contrast to the specificity of adenine compound deaminases of animal tissues.

McElroy and Mitchell ('46) in a biochemical genetic study attempted to detect some difference in the adenosine deaminase activity of cell-free extracts prepared from wild-type

and temperature-sensitive (adenine requirement at 35°C., none at 25°C.) strains of *N. crassa*. Adenosine was rapidly deaminated by both strains. No adenase activity could be detected.

DEAMINATION OF ADENINE AND ADENOSINE BY ESCHERICHIA COLI

Lutwak-Mann ('36) described experiments in which suspensions of *E. coli* deaminated adenine and adenosine. Phosphate and arsenate caused an increased rate of deamination. This activation apparently has not been further investigated by any one.

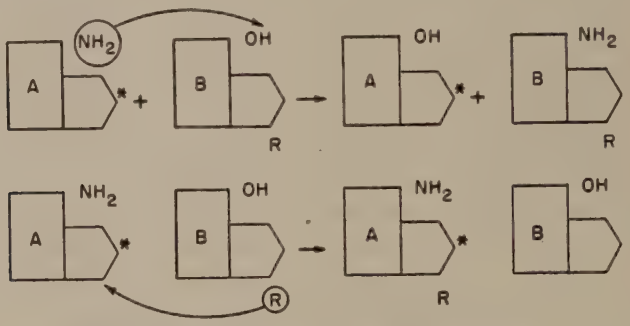

Fig. 1 Transaminase vs. *trans*-N-glycosidase.

Stephenson and Trim ('38) made the interesting observation that the rate of deamination of adenine by *E. coli* suspensions was increased six to seven times by the presence of a small amount of adenosine. The catalytic effect of adenosine on the deamination of adenine fell off with time. If fresh adenosine was added the initial rate was again restored. Stephenson and Trim suggested as one possibility a transamination involving a transfer of the NH_2 group from adenine to inosine with the formation of hypoxanthine and adenosine. The adenosine upon deamination forms inosine which again can accept the NH_2 group from adenine and so on (fig. 1).

Kalckar ('50) was very much interested in the Stephenson-Trim finding for it suggested a new route for enzymatic syn-

thesis of adenosine, a route which could explain the initial steps necessary for the rapid incorporation of labeled adenine into nucleic acid (Brown et al., '48). It should be pointed out that adenine is completely inactive as a substrate for nucleoside synthesis via purine nucleoside phosphorylase, a point which had been of some concern to Kalckar.

Kalckar was intrigued with the idea that the catalytic effect of adenosine on adenine deamination could be explained by a *trans*-N-glycosidase type of reaction as well as by a transamination. A transfer of ribose would give the same products as a transfer of NH_2, adenine plus inosine yielding hypoxanthine and adenosine in each case (fig. 1).

Proof for the existence of a *trans*-N-glycosidase reaction was soon forthcoming in the work of MacNutt ('52). The transfer reaction will be discussed in more detail later, but we would like to describe briefly some recent experiments of Kalckar, MacNutt, and Hoff-Jørgensen ('52) at this point, for they trace back to the Stephenson-Trim deamination findings.

A direct exchange of C^{14}-adenine with the hypoxanthine of hypoxanthine desoxyriboside was shown to be catalyzed by cell-free extracts of *Lactobacillus helveticus*. An analogous reaction with inosine, however, was negative. No formation of C^{14}-adenosine, C^{14}-inosine, or C^{14}-hypoxanthine could be detected. One possible explanation for the negative results was that a very active purine nucleoside hydrolase split adenosine but not adenine desoxyriboside. This explanation finds some support in the findings of Wang and Lampen ('51) which show that cell-free extracts of *L. pentosus* rapidly split ribonucleosides but not the desoxyribonucleosides.

Kalckar, MacNutt, and Hoff-Jørgensen found that the *L. helveticus* extracts could deaminate adenosine but not adenine desoxyriboside, in contrast to the action of mammalian adenosine deaminase as well as the deaminase of *E. coli* and *L. casei*.

Guanosine deaminase. Lutwak-Mann ('36) very briefly mentioned that guanosine was deaminated in the presence

of phosphate by suspensions of *E. coli*. I know of no other work with microbial enzymes.

Cytosine nucleoside deaminase. Wang et al. ('50) have prepared cell-free extracts from *E. coli* and brewers' yeast which convert cytidine to uridine with the release of 1 mole of ammonia per mole of nucleoside. The deamination was conveniently followed by differential spectrophotometric methods. The purified *E. coli* preparation attacked the riboside and desoxyriboside of cytosine only, having no deaminase action on adenine, adenosine, cytosine, isocytosine, guanine, and guanosine.

Paege and Schlenk ('50) found that suspensions of *E. coli* and *A. aerogenes* would deaminate cytidine and cytidylic acid, the specificity of the deamination not being determinable under these conditions.

Cytosine deaminase. Chargaff and Kream ('48) have demonstrated the presence of cytosine deaminase in cell-free extracts of *E. coli* and yeast. The yeast enzyme (Kream and Chargaff, '50) was purified fivefold. Guanase and adenase were not present. 5-Methyl cytosine was deaminated to thymine. Cytidine and cytidylic acid were not attacked.

Wang and Lampen ('52a) described experiments with resting cells of a soil bacterium which deaminates cytosine to uracil under anaerobic conditions. This activity however could not be found in cell-free extracts (Wang and Lampen, '52b).

Nucleoside hydrolases (table 2). Wang and Lampen ('51) found that cell-free extracts of *L. pentosus* contained nucleosidases which split adenosine, cytidine, xanthosine, guanosine, inosine, and uridine. Adenosine and cytidine were split directly to free adenine and free cytosine without any previous deamination. There were indications that the bacterial nucleosidases were different from the mammalian nucleoside phosphorylases: (1) the splitting of adenosine and cytidine is not an activity of nucleoside phosphorylase, and (2) the ribosides of hypoxanthine, cytosine, and uracil were rapidly split whereas the corresponding desoxyribosides were very

slowly split, if at all, by the bacterial enzymes. Ribose-1-phosphate was not split by the extracts. A mixture of uridine or cytidine with ribose-1-phosphate however gave free pentose (Lampen, '51; Lampen and Wang, '52). It appeared therefore that the splitting was a hydrolytic irreversible process.

Carter ('51) has partially purified an enzyme from plasmolyzed yeast which rapidly splits uridine in the absence of phosphate or arsenate. Adenosine, inosine, guanosine, cytidine, and thymidine are not degraded.

TABLE 2

Nucleoside hydrolases

SUBSTRATE	ORGANISM	REFERENCE
1. Cytidine Adenosine Xanthosine	*L. pentosus*	Wang and Lampen, '51
2. Uridine	Yeast	Carter, '52
3. Adenosine Inosine Guanosine Nicotinamide riboside Xanthosine Synthetic purine nucleosides	Yeast	Heppel, '52
4. Inosine	*L. helveticus* *delbrueckii* Thermobacter species	Kalckar et al., '52

Heppel ('52) has recently accomplished a separation of two types of nucleosidase activity in yeast. One activity is very much like that of mammalian purine nucleoside phosphorylase. The other activity is a hydrolytic activity which Heppel has named "purine nucleoside hydrolase." The hydrolase splits adenosine, guanosine, inosine, nicotinamide riboside, and certain synthetic nucleosides including 2-aminoadenosine. Uridine and cytidine are not split. The hydrolysis is an irreversible reaction which goes to completion with the release of free ribose and nitrogenous base. Ribose-1-phosphate is not split by the enzyme preparation. The cleavage

of adenosine is not preceded by deamination. There is no requirement for phosphate or arsenate. The possibility of an exchange reaction similar to MacNutt's desoxyriboside transfer mechanism was investigated with C^{14}-adenine and inosine and no evidence for exchange could be demonstrated.

Kalckar et al. ('52) also observed nucleoside hydrolase activity in extracts of *L. helveticus, delbrueckii* and in *Thermobacter* sp.

Phosphatases. Nonspecific nucleophosphatase activities have been reported to be present in many microorganisms. However, information on specific microbial nucleophosphatases does not appear to be available.

Purine and pyrimidine dehydrogenases. Krebs and Eggleston ('39) prepared cell suspensions of *Corynebacterium* grown on a medium containing uric acid, which would convert uric acid, allantoin, and allantoic acid to urea. Hypoxanthine was also oxidized, but more slowly.

Barker and Beck ('41) described an anaerobic dehydrogenase system present in *Clostridium acidi-urici* which apparently decomposed uric acid in a completely different way. The products of this dehydrogenation were ammonia, acetic acid, and carbon dioxide. Xanthine, guanine, and hypoxanthine could also serve as purine substrates for the decomposition. Allantoin and uracil were not attacked.

The decomposition of hypoxanthine leads to the formation of more acetic acid than can be accounted for by the direct decomposition of the C_3 chain in the purine structure. Part of the acetic acid must therefore come from more complex condensations. Glycine is metabolized when purine is present and only when purine is present. For example the amount of ammonia released from a mixture of uric acid and glycine is in excess of that which could arise from uric acid decomposition alone. It has been postulated that a purine intermediate of unknown nature acts as a hydrogen acceptor from glycine.

Tracer experiments with labeled uric acid (Karlsson and Barker, '49) reveal that the 2 and 8 positions contribute almost half of the methyl carbon of acetic acid. A fixation of

carbon dioxide occurs with conversion to acetic acid. Karlsson and Barker have concluded from their isotopic tracer experiments with the *Clostridium*-uric acid system that further elucidation can probably best be achieved by enzymatic methods. More work with this very interesting organism should be carried on.

Wang and Lampen ('52a, b) have prepared cell-free extracts from a soil bacterium which, in the presence of methylene blue and air, catalyze the oxidation of uracil and thymine. Barbituric acid was isolated as an oxidation production of uracil.

Hayaishi and Kornberg ('51) have also obtained barbituric acid from uracil oxidation by strains of *Corynebacterium* and of *Mycobacterium*. 5-Methyl barbituric acid was believed to be the oxidation product of thymine.

Hayaishi ('52) is reporting at the Federation meetings that he has isolated an enzyme from uracil-adapted cells which catalyzes the further conversion of barbituric acid to urea and malonic acid. 5-Methyl barbituric acid is not attacked by the enzyme preparations.

NUCLEOSYNTHETIC ENZYMES

Nucleoside phosphorylase. In the Kalckar reaction (Kalckar, '47) the reversible phosphorolysis of nucleosides results in the formation of ribose-1-phosphate or desoxyribose-1-phosphate. Nucleoside synthesis is generally favored rather than the release of free base. This is quite opposite to the situation with the hydrolases where splitting of the nucleosides goes to completion and is irreversible.

Ribose-1-phosphate as a crystalline potassium salt has recently been prepared by Webster of the Department of Biochemistry at Washington University. Desoxyribose-1-phosphate was obtained as a crystalline cyclohexylamine salt a few years ago in Kalckar's laboratory (Friedkin, '50).

The phosphorolytic reactions were first encountered in mammalian tissues. With regard to these reactions in microorganisms, Lutwak-Mann ('36) and later Stephenson and

Trim ('38), in studies already mentioned in connection with the deaminases, found that adenosine was split and its ribose utilized by suspensions of *E. coli.*

Manson and Lampen ('51) found that *E. coli* contains enzymes which split desoxyribonucleosides to the free base and a desoxyribose ester. The acid lability of the ester pointed to a phosphorylation at the 1 position of desoxyribose. Cell-free extracts catalyzed interconversions between the desoxyribonucleosides of thymine, uracil, and hypoxanthine. The desoxyribonucleosides of adenine and cytosine were not attacked.

Paege and Schlenk ('50; Schlenk '51) have isolated and purified an enzyme from *E. coli* which splits uridine with the formation of ribose-1-phosphate. Cytidine and purine ribosides are not attacked.

Sable ('50) has reported the presence of purine nucleoside phosphorylase in yeast extracts. We have already mentioned the recent work of Heppel ('52) in which yeast purine nucleoside phosphorylase and hydrolase have been separated. Inosine, guanosine, and nicotinamide riboside are split by Heppel's yeast nucleoside phosphorylase. The reaction requires inorganic phosphate and results in its esterification. Nucleoside synthesis was demonstrated with ribose-1-phosphate and hypoxanthine.

In general, the specificity of the microbial nucleoside phosphorylases seems to be similar to that of the mammalian enzymes (fig. 2). Separate enzymes exist for the splitting of purine and pyrimidine nucleosides. Adenosine and cytidine are uniquely inactive in these systems. Some of the reactions involving xanthine (Friedkin, '52a), 8-azaguanine, 5-aminouracil, and 2-thiouracil (Friedkin, '52b) have not been demonstrated with the microbial nucleoside phosphorylases but this can probably be done.

Desoxyribosyl-trans-N-glycosidase. MacNutt ('52) in an attempt to explain why different desoxyribonucleosides can act as growth factors for certain strains of vitamin B_{12}-requiring lactic acid bacteria, investigated cell-free extracts

of *L. helveticus* for the presence of nucleoside phosphorylase activity. Much to his surprise, none could be demonstrated. The extracts did not catalyze the formation of a nucleoside from desoxyribose-1-phosphate plus a purine or pyrimidine. What MacNutt did find was a reaction in which any one of many desoxyribonucleosides appeared to form an intermediate desoxyribosyl enzyme complex which then could transfer the desoxyribosyl group to other purines or pyrimidines.

The specificity of the MacNutt reaction is much broader than that of the phosphorylases, with transfers of desoxy-

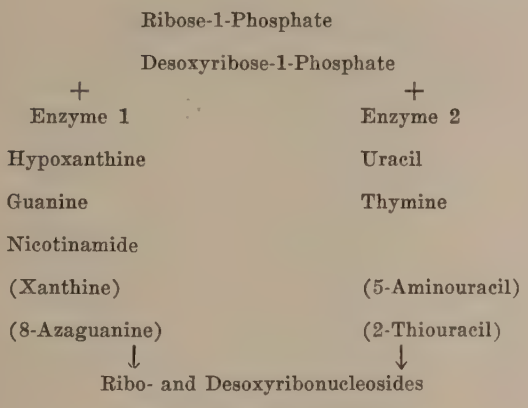

Fig. 2 Kalckar reaction.

ribosyl possible to adenine, cytosine, 5-methylcytosine, and 4-amino-imidazole-5-carboxamide (fig. 3). Unfortunately, orotic acid was not tested. The ability to transfer desoxyribosyl to inorganic phosphate with the formation of desoxyribose-1-phosphate is not present in the *L. helveticus* enzyme preparations.

Hoffman ('52) is reporting at the Federation meetings that the enzymes of *E. coli* formerly thought to be nucleoside phosphorylases are also transferring enzymes, one enzyme catalyzing the direct transfer of desoxyribose from pyrimidine to pyrimidine and the other from purine to purine. Each of the enzymes can transfer desoxyribose to inorganic phosphate,

the desoxyribose-1-phosphate thus formed being the connecting link between the two systems.

This does not appear to be the case with purified pyrimidine nucleoside phosphorylase prepared from horse liver (Friedkin, '52, unpublished experiments). There is absolutely no incorporation of inorganic P^{32} into esterfied phosphate when desoxyribose-1-phosphate is incubated with the mammalian enzyme. Only when small amounts of acceptor are added, thymine or uracil, does turnover occur. Thus the situation with mammalian pyrimidine nucleoside phosphorylase ap-

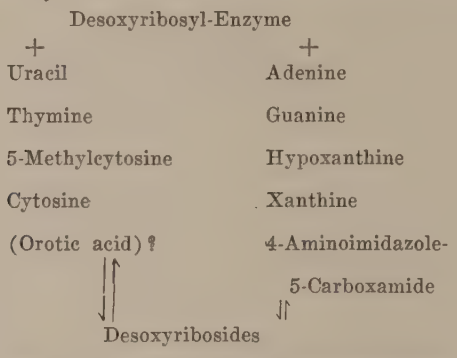

Fig. 3 MacNutt reaction in *Lactobacillus helveticus.*

pears to be similar to that of muscle glycogen phosphorylase (Cohn and Cori, '48).

Adenosine phosphokinase. Sable ('50), Caputto ('51), and Kornberg and Pricer ('51) have prepared an enzyme from brewers' yeast which catalyzes the direct phosphorylation by ATP of adenosine. 2,6-Diaminopurine riboside can also serve as a substrate for the phosphorylation. A similar reaction has not been demonstrated for any of the other nucleosides.

IMPORTANT LEADS

Most of this discussion has concerned intact cells which degrade various substrates and the enzymatic activities of cell-free systems. Sometimes, due to environmental or genetic factors which upset the normal metabolic patterns,

intermediates accumulate which are not usually encountered. Such intermediate accumulation has supplied important leads toward a fuller understanding of purine and pyrimidine metabolic pathways.

The formation of purple pigment in adenine-requiring *Neurospora* mutants (Mitchell and Houlahan, '46) and the accumulation of 4-amino-imidazole-5-carboxamide in sulfonamide-inhibited *E. coli* (Stetten and Fox, '45; Shive et al., '47) are examples of this phenomenon in purine metabolism.

The formation of orotic acid (Mitchell et al., '48) and its riboside (Michelson et al., '51) in uridine-requiring *Neurospora* mutants and the accumulation of uracil compounds in penicillin-treated *Staphylococcus aureus* (Park, '52a, b, c) provide unique leads in pyrimidine metabolism and are the subject of intensive research programs in many laboratories.

EXPERIMENTS WITH TETRAHYMENA

Recently we have turned to *Tetrahymena geleii* [3] as a source of nucleosynthetic enzymes. These protozoa are very easy to grow, they multiply rapidly, and their cell walls are quite fragile, making it a simple matter to prepare extracts (Kidder and Dewey, '51). We have been particularly interested in the enzymes involved in desoxyribonucleic acid synthesis and hope that the *Tetrahymena* will prove to be useful in this direction.

Washed *Tetrahymena* cells have been homogenized with isotonic sucrose and, after high speed centrifugation of the homogenates, cell-free supernatant fractions have been obtained. The extracts show very high pyrimidine nucleoside phosphorylase activity. The extracts have been fractionated with ammonium sulfate, most of the pyrimidine nucleoside phosphorylase activity appearing in protein fractions precipitable by 2 *M* ammonium sulfate.

The mode of thymidine splitting has been of particular interest to our group, for it could be due to hydrolytic, phos-

[3] We are very grateful to Dr. George W. Kidder, Amherst College, for a culture of strain W of *Tetrahymena geleii* and for his many kind suggestions relating to the handling of these organisms.

phorolytic or transferring activity. Thymine conceivably may be released from thymidine either by an arsenolysis (Manson and Lampen, '49) or by an exchange with a purine such as adenine (MacNutt, '52):

Thymine desoxyriboside + arsenate ⇌ thymine + (desoxyribose-1-arsenate) → desoxyribose

Thymine desoxyriboside + adenine ⇌ thymine + adenine desoxyriboside

TABLE 3

Mechanism of thymidine splitting by Tetrahymena and horse liver enzymes

ADDITIONS	RELEASE OF FREE THYMINE			
	Tetrahymena enzyme		Horse liver enzyme	
	− Arsenate	+ Arsenate	− Arsenate	+ Arsenate
	Increase of optical density at wave length 300 mμ			
None	0.017	0.231	0.000	0.170
Adenine	0.012	0.237	0.001	0.168
Hypoxanthine	0.010	0.226	— 0.001	0.175

TABLE 4

Enzymatic synthesis of desoxyribosides of thymine and uracil with Tetrahymena

TIME OF INCUBATION AT 38°C.	RELEASE OF INORGANIC PHOSPHATE		
	Desoxyribose 1-P alone	Desoxyribose 1-P and thymine	Desoxyribose 1-P and uracil
Min.	*μM*	*μM*	*μM*
0	0.013	0.014	0.015
28	0.032	0.112	0.131
60	0.086	0.168	0.196

Thymidine was incubated in the presence and absence of arsenate (table 3). Arsenate greatly stimulated the release of thymine, determined as the increase of optical density at a wave length 300 mμ in alkaline medium. Adenine and hypoxanthine were added in the presence and absence of arsenate. In the absence of arsenate there was no stimulation of thymine release upon the addition of the purines. The presence of purines, moreover, was not inhibitory to the arsenolysis.

If the MacNutt transfer mechanism had been operative here, the addition of the purines should have stimulated thymine release. This was not the case. For comparison, similar data were obtained with purified pyrimidine nucleoside phosphorylase from horse liver.

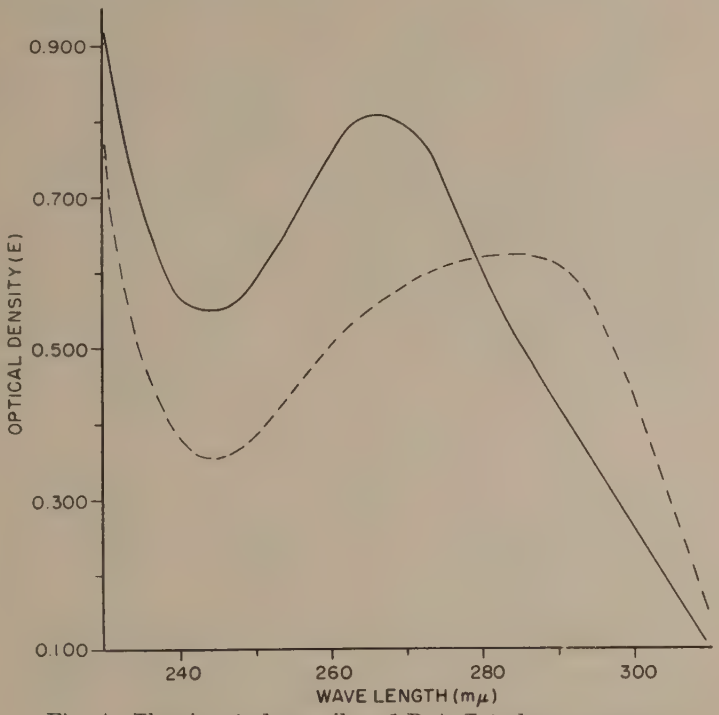

Fig. 4 Thymine + desoxyribose-1-P + *Tetrahymena* enzyme.
——— 60 minutes – – – – zero time

Tests were then made with the *Tetrahymena* enzyme for pyrimidine nucleoside synthesis (Friedkin and Roberts, '51). A slow splitting of desoxyribose-1-phosphate occurred when this substrate was incubated alone with the *Tetrahymena* preparation (table 4). If thymine or uracil were also present, inorganic phosphate was released at an accelerated rate, indicating nucleoside synthesis.

The spectra in alkaline medium of aliquots taken from the incubation mixtures of desoxyribose-1-phosphate, thymine, or

uracil and *Tetrahymena* enzyme were determined. The spectra indicated a conversion of 37% of the thymine initially present to thymidine (fig. 4). Similarly, with uracil as a substrate, 32% conversion to uracil desoxyriboside occurred (fig. 5). Approximately 1 mole of pyrimidine desoxyriboside

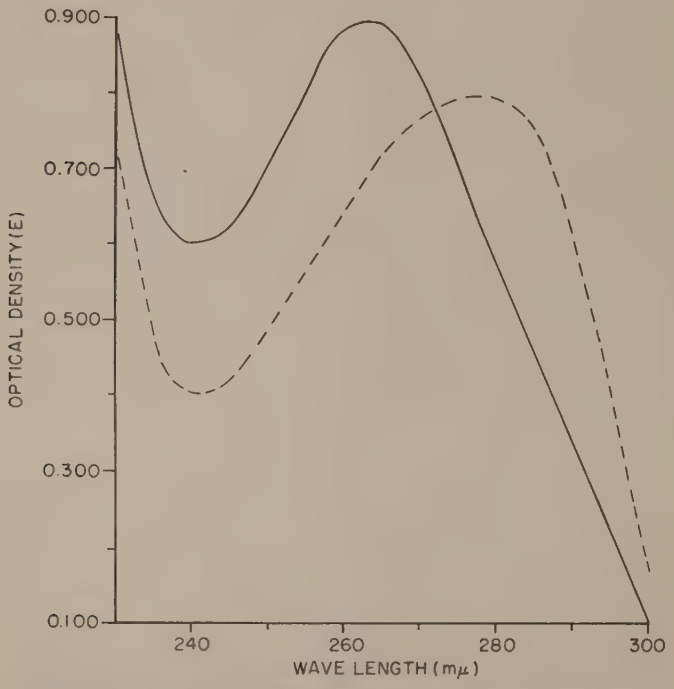

Fig. 5 Uracil + desoxyribose-1-P + *Tetrahymena* enzyme.
——— 60 minutes - - - - zero time

was formed per mole of inorganic phosphate released from desoxyribose-1-phosphate.

We conclude from these experiments that *Tetrahymena* contain a pyrimidine nucleoside phosphorylase. The transferring enzyme may also be present but we have not detected it.

In closing I would like to suggest that it is much too early to integrate the available data into a satisfactory system of

reactions which represents the main pathway of nucleic acid synthesis.

DISCUSSION

LAMPEN: Have you tried this last system that you were running, with the possibility that you might have one of the transfer systems that we had in coli?

FRIEDKIN: After seeing your abstract, I thought that I should try it but did not have time.

HORECKER: I wish to raise an objection to referring to the nucleoside hydrolysis as irreversible, because after all, with the concentration of water that is present in the medium compared with the concentration of phosphate in the phosphorolysis reaction, it may very well be that the equilibrium constants for those two reactions are nearly the same. In the presence of such an excess of water, it may not be possible to detect the reversal.

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